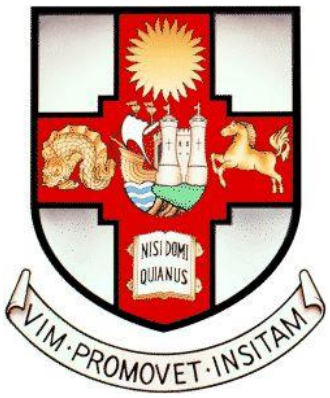
The management of UK pasture-fed beef production systems and impacts on dung and disease.



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Abstract

This thesis places dung firmly at the centre of livestock farming. It tests the overarching hypothesis that intentional management of dung as a critical resource on-farm has multiple benefits that improve the resilience of beef production systems.

Dung is a primary resource in beef production systems. This importance of dung as an organic fertiliser that recycles nutrients in agricultural systems is widely recognised and understood. The connection between disease, dung and its management through diet choice for beef cattle has received far less attention.

In this study, the cattle diet, both intake and forage management, significantly influenced the rate of bulk and biochemical (fibre fractions, protein, lipids, carbohydrates, organic matter, ash and micronutrients) dung degradation over 84 days under three forage management treatments on the North Wyke Farm Platform. Site-specific factors were the primary drivers of bulk dung degradation; 70% of degradation was driven by field site and 4% by dung type.

Non-target impacts of anthelmintics pose a risk to dung fauna that moderate dung degradation. A multiple-regression model predicted that targeted selective treatments (TST) created refugia for dung-breeding fly *Scathophaga stercoraria* and that proportions of treated cattle (55%) was more influential than effective dung drug concentrations (13%).

Effective TST programmes rely on practical diagnostic tools. A non-invasive protocol was developed for the quantification of immuno-markers (immunoglobulins and lactoferrin) in dung for gastrointestinal health assessment.

The anecdotal benefits of increasingly popular ‘mob grazing’ were supported by case studies of factors including pasture performance, reduced gastrointestinal nematode (GIN) burden, and soil organic matter content over a grazing season in a UK-wide study.

The conclusion of this multidisciplinary study is that dung management has a critical role to play in a systems-level understanding of beef production and that optimisation relies on a quantitative understanding of the relationships between wider biological processes on farms.

Dedication and Acknowledgements

Dedications

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Final thanks go those at Rothamsted Research, North Wyke, past and present, for their support and advice over the years.

Author's declaration

I declare that the work in this dissertation was carried out in accordance with the requirements of the University's Regulations and Code of Practice for Research Degree Programmes and that it has not been submitted for any other academic award. Except where indicated by specific reference in the text, the work is the candidate's own work. Work done in collaboration with, or with the assistance of, others, is indicated as such. Any views expressed in the dissertation are those of the author.

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Glossary of terms

ADF – acid detergent fibre

ADL – acid detergent lignin

ADS – acid detergent solution

AHDB - Agriculture and Horticulture Development Board

AMR – antimicrobial resistance

CarLA – Carbohydrate larval antigen

CCA – Circulating cathodic antigen

CL – crude lipids

CP – crude protein

DEFRA - Department for Environment, Food & Rural Affairs

DM – dry matter

EC – effective drug dung concentration

EDTA – Ethylenediaminetetraacetic acid

epg – egg(s) per gram

ELISA – enzyme-linked immunosorbent assay

EU – European Union

FAD – faecal antibody detection

FAMACHA - Faffa Malan Chart

FAO – Food and Agricultural Organization

FEC – faecal egg count

GIN – gastrointestinal nematode

GPS – global positioning satellite

HCl – hydrochloric acid

HRP – horseradish peroxidase

Ig – immunoglobulin

IgA – immunoglobulin A

IgD – immunoglobulin D

IgE – immunoglobulin E

IgG – immunoglobulin G

IgM – immunoglobulin M

LOD – limit of detection

NDF – neutral detergent fibre

NDS – neutral detergent solution

NFC – non-fibre carbohydrates

NIRS – near-infrared spectroscopy

NVZ – nitrate vulnerable zone

OD – optical density

OM – organic matter

PCR – polymerase chain reaction

PT – proportion treated

SOM – soil organic matter

TB - tuberculosis

TBST – tris buffer saline solution with tween

TMB - 3,3',5,5'-Tetramethylbenzidine

TST – targeted selective treatment

UK – United Kingdom

XRF – X-ray fluorescence

Chapter 1

Literature review – Challenges of pasture-based beef production

Summary

The focus of innovation in beef production worldwide has been the increase in liveweight gain, and with less attention paid to improving other aspects of the livestock system. Beef is a high-status food, with demand and rates of consumption growing globally. To meet this demand in an environmentally appropriate manner, all aspects of the production cycle must be optimised. Achieving this goal requires a multi-disciplinary approach to identify and address areas for improvement.

With over 1.5 billion cattle worldwide, beef production has major significance economically, environmentally, nutritionally, and culturally. Within such systems, dung plays a crucial role in the turnover of nutrients, emission of greenhouse gasses, and the transmission of parasitic diseases. Despite this, there is remarkably little consideration placed on the specific characteristics of dung and how it can be more effectively considered and utilised as a resource within livestock systems. The influence of dung is large and widespread, impacting pasture fertility, greenhouse emissions, pathogen epidemiology, while also being able to provide valuable information about animal health. Therefore, enhancing our knowledge and capabilities to effectively utilise dung as a resource represents an opportunity to improve the way in which livestock production systems function. The scale and distribution of beef production means that its potential impact is high and that even the smallest of changes to production can have a big effect. Due to the complex and dynamic nature of livestock systems, advancement requires multi-disciplinary and multi-level scientific research.

1.1 Dung

Dung is a fundamental and valuable resource in pasture-based livestock systems and is involved in a host of processes. The impact of dung can be both positive and negative, depending on the metric and context considered. From promoting pasture productivity to facilitating parasitic diseases, the importance of dung within grazing livestock systems cannot be underestimated. Factors surrounding the role and characteristics of cattle dung are extremely interlinked and therefore complex. There are 1.5 billion cattle on the globe (Figure 1.1) that produce substantial quantities of faeces. According to Lorimor et al. (2004), a 450kg beef cow will produce approximately 15 tonnes of dung per year, while a 200kg calf may produce 8 tonnes. At the global-scale, this equates to annual production of an estimated 15-20 billion tonnes. Therefore, a minor modification in dung management could have significant consequences.

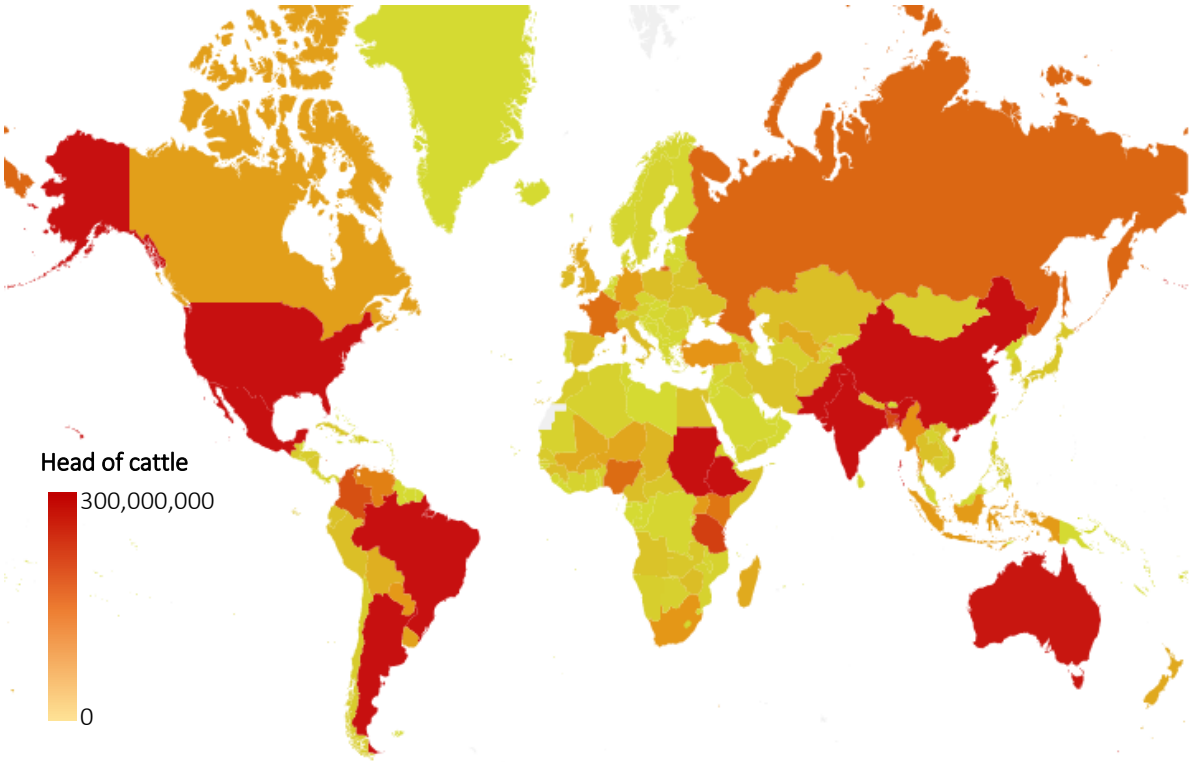


Figure 1.1 - Heat map of worldwide head of cattle by country. The country with the least is Greenland (15), and the country with the most is Brazil (211 million). No data was available for the Western Sahara (white). Source: original image, created using data from FAO (2017).

Recent debates about the sustainability of meat in human diet has focussed on the human health and environmental impacts, especially the contribution to greenhouse gas emissions

and climate change caused by methane production during ruminant fermentation (Garnett, 2009; Gill et al., 2010; Phetteplace et al., 2001), with comparatively minor flux from dung and urine (Flessa et al., 2002; Schils et al., 2007). A 2006 report by the Food and Agricultural Organization of the United Nations, entitled *"Livestock's long shadow*" (Steinfeld et al., 2006), brought attention to the environmental impact of livestock and calculated that livestock production was responsible for 18% (later revised to 15% (Gerber et al., 2013)) of global greenhouse gas emissions. Such reports have added to the pressure to reduce global consumption of and the environmental footprint of beef, although quantity and price remain the primary factors considered when purchasing beef by the majority of consumers (Verbeke et al., 2010). This creates an issue for beef producers, particularly those who place value on environmental sustainability and manage their farm accordingly. While short-term profitability and long-term environmental sustainability often contrast each other, that need not be the case. For instance, effective land and grazing management improve indicators of soil health, e.g. soil carbon sequestration when compared to arable systems (Lal, 2004; Freibauer et al., 2004). Dung can significantly increase soil microbial biomass and respiration (Belay et al., 2001; Ghoshal and Singh, 1995; Lovell and Jarvis, 1996; Rochette and Gregorich, 1998; Witter et al., 1993), two primary metrics representative of soil quality, health, and productivity (Rice et al., 1996; Schloter et al., 2003). By improving soil health, the need for external fertiliser inputs is lessened (Aarons et al., 2009; Ayoola and Makinde, 2008), reducing costs and potentially the run-off impacts such as water-course eutrophication (Weimin and Lijiao, 2001).

1.1.1 Nutritional value

A key role of dung is as an organic fertiliser that facilitates the cycling of nutrients from cattle diet back into the soil system to provide nutrients for the subsequent crop, and secondarily a vast variety of organisms that rely on dung within their food web (Aarons et al., 2009; Ayoola and Makinde, 2008; During et al., 1973; Moe and Wegge, 2008; W. Sheldrick et al., 2003; Williams and Haynes, 1995). Dung is as a source of nutrients within the wider environment, acting both a food source and refuge for invertebrates and microorganisms (Dungait et al., 2008; Marshall, 1977; Standen, 1984). These, in turn, can help to facilitate nutrient cycling while also enhancing local ecology and system health (D'arcy-Burt and Blackshaw, 1991; McCracken et al., 1995).

1.1.2 Dung degradation

The rate at which dung degrades is influenced by a plethora of interacting factors, making it a complex process, such factors include: fauna, climate, use of veterinary drugs, and diet. Small changes in these variables have the potential to alter the rate of degradation greatly. It should be noted that many of the factors which influence dung degradation do so, at least in part, due to their effect on the composition of fauna that colonise the dung and which are instrumental in the process of degradation.

1.1.2.1 Climate and weather

The role of climatic variables upon dung degradation has been well documented, particularly the influence of gross seasonal changes (Dickinson et al., 1981; Holter, 1979; Weeda, 1967). However, due to the complex interactions between climate and dung degradation, it is important to understand the individual factors involved. Post-excretion, the water content of dung is mainly controlled by climatic factors, notably, precipitation and temperature, but potentially also humidity and sunlight. Dickinson et al. (1981) reported rainfall to be a main climatic factor influencing degradation, due to the physical breakdown of dung by the water and by the increases in dung water content. Weeda (1967) found that consistency of dung due to liquid content could increase degradation by 100% when comparing the moistest natural dung to the driest. Further evidence in support was found by Barth et al. (1995), who reported that small changes in dung moisture of just 1-2% could significantly alter dung fauna development and dung degradation. Increased air temperatures have the potential to reduce dung water content through evaporation, however, after a point and especially with radiative heat from sunlight, dung may form an exterior crust. This crust protects the inside of the pat and can help to retain moisture (Dungait et al., 2005; Holter, 1979). At the opposite end of the spectrum, low temperatures may freeze the dung, this locks up water, reducing its availability, it also protects the dung from physical breakdown. At such temperatures, enzymatic activity in soil microbes would decrease, and the invertebrate community would be greatly reduced. Climate may also indirectly affect diet and excreted nutrients through its impacts upon flora communities, water availability and utilisation, feeding patterns, and energy expenditure.

1.1.2.2 Diet and nutrition

There has been limited research linking animal diet, dung composition, and dung degradation. The physical composition of cattle dung is determined by the animal's biology, diet, and utilisation of nutrients. The varied and significantly different diets of different cattle may result in significant differences in the composition of their dung and therefore impact upon all dung related processes including invertebrate activity, degradation, nutrient composition, and gastrointestinal nematode (GIN) activity. Barth (1993) observed that cattle diet was a driver of invertebrate colonisation of dung while further research by (Barth et al., 1994b) found that specific dung factors such as pH, moisture, and organic matter content, influenced beetle colonisation behaviour and development. Diet has also been found to impact the faecal excretion of veterinary anthelmintics, administered to cattle, with dung ivermectin residues being five times greater from grain-fed cattle than from grazing cattle (Cook et al., 1996). The same study also found that grain-fed cattle produced more acidic dung than grazing cattle (6.4 and 7.3 respectively). These results highlight the impact that dung composition can have upon a variety of different dung processes. The general lack of research into this means it is a key area in which understanding can be enriched, with the potential to inform positive changes in beef production systems. Achieving this requires clearly defined methodologies in dung analysis.

1.1.2.3 Anthelmintic use

Many invertebrates are instrumental in the breakdown of dung and its incorporation into the soil. Thus a reduction in populations may reduce the rate of dung degradation. As previously discussed, the application of anthelmintics, for the control of endoparasites, can significantly impact the rate by which cattle dung degrades, impacting the nutrient cycle and local ecology (Barth et al., 1993; Floate, 1998a). The primary consideration for the use of anthelmintics is, as it should be, animal health and wellbeing. However, there is scope to include consideration of the environmental impacts of anthelmintics as part of wider veterinary strategies, complementing the veterinary evidence supporting restricting anthelmintic use.

1.1.3 Dung and parasites

Dung is involved in the lifecycle and transmission of numerous significant livestock pathogens. This is particularly true of parasitic diseases such as GINs, whose eggs are excreted in the

faeces of their host. The dung then provides a warm, moist, and protected environment for the eggs and larvae to develop, after which they migrate onto pasture to infect a host. Dung fauna can also play a significant role in the control of GIN populations, through the consumption and burying of dung, killing or inhibiting larvae (Fincher, 1973, 1975). This also further highlights the importance of considering the non-target, insecticidal, impact of anthelmintics excreted within dung. The timing and distance over which migration occurs is a key lifecycle variable, and there are many factors which influence this, including those which are involved in dung degradation. Soil type affects larval migration (Stromberg, 1997) and it is thus reasonable to consider dung consistency, as determined by climate and diet may also. If migration from the dung onto pasture requires increased energy and time, it is reasonable to believe that that will reduce the distance which the parasite may migrate away from the dung. This is particularly important because cattle avoid grazing near faeces. To add further complexity: as dung degrades and is influenced by its environment, the consistency and size will change, along with the parasites ability to migrate out of it. Within dry faeces, parasites may be desiccated, and movement inhibited, this may force migration downwards into the soil, while moisture provides a medium for larvae to move in. Rain may wash larvae onto surrounding herbage, and heavy droplets can transport larvae as far as 90cm (Stromberg, 1997). As with all organisms, each parasite has an optimum temperature, at which its fitness is highest, deviation from this temperature will lead to inhibition of metabolic process and thus the ability of larvae to exit the faeces.

Irrespective of international differences in beef production systems, the ubiquity and scale of beef production means that small changes can have a large impact. The complex and dynamic nature of livestock production systems means that there is a vast array of factors which can influence production and impact. Whether it be aspects of animal health, fertiliser use, soil quality, or other, no single factor is of most importance, and no single factor sits in isolation. It is, therefore, necessary to take a multi-disciplinary and multi-level approach to researching livestock production for its long-term development and sustainability.

Dung has another, highly significant, role within agriculture. Dung is a vehicle for the expulsion of the eggs of parasitic gastrointestinal nematodes (GINs) (and other gastrointestinal helminthic parasites) and is an essential part of the GIN lifecycle (Figure 1.2). As a host, cattle provide a relatively stable, albeit challenging, environment for GINs, as does the warmth and

moisture of dung. Nematodes (of all types) are found throughout the lithosphere (Borgonie et al., 2011) with the widely reported figure that nematodes represent up to 80% of all individual animals on earth. Whether or not that figure is correct, it highlights their ubiquity and their ability to adapt and exploit resources, including other animals. This ubiquity, combined with the worldwide distribution of cattle, this means that GINs and associated diseases are prevalent worldwide. This is compounded by the increasing occurrence of anthelmintic resistance and represents a significant threat to livestock production (Waller, 1994, 1997, 1999). Stemming the tide of anthelmintic resistance requires changes in the way in which we treat and prevent GIN infections. Successful implementation of resistance mitigating treatment strategies, such as targeted selective treatment (TST) (Kenyon et al., 2009; van Wyk et al., 2006) requires an advancement in veterinary diagnostics. While technologies for the molecular and immunological diagnosis of health are routinely used within human medicine, the practical translation of these technologies into effective veterinary tools has lagged far behind.

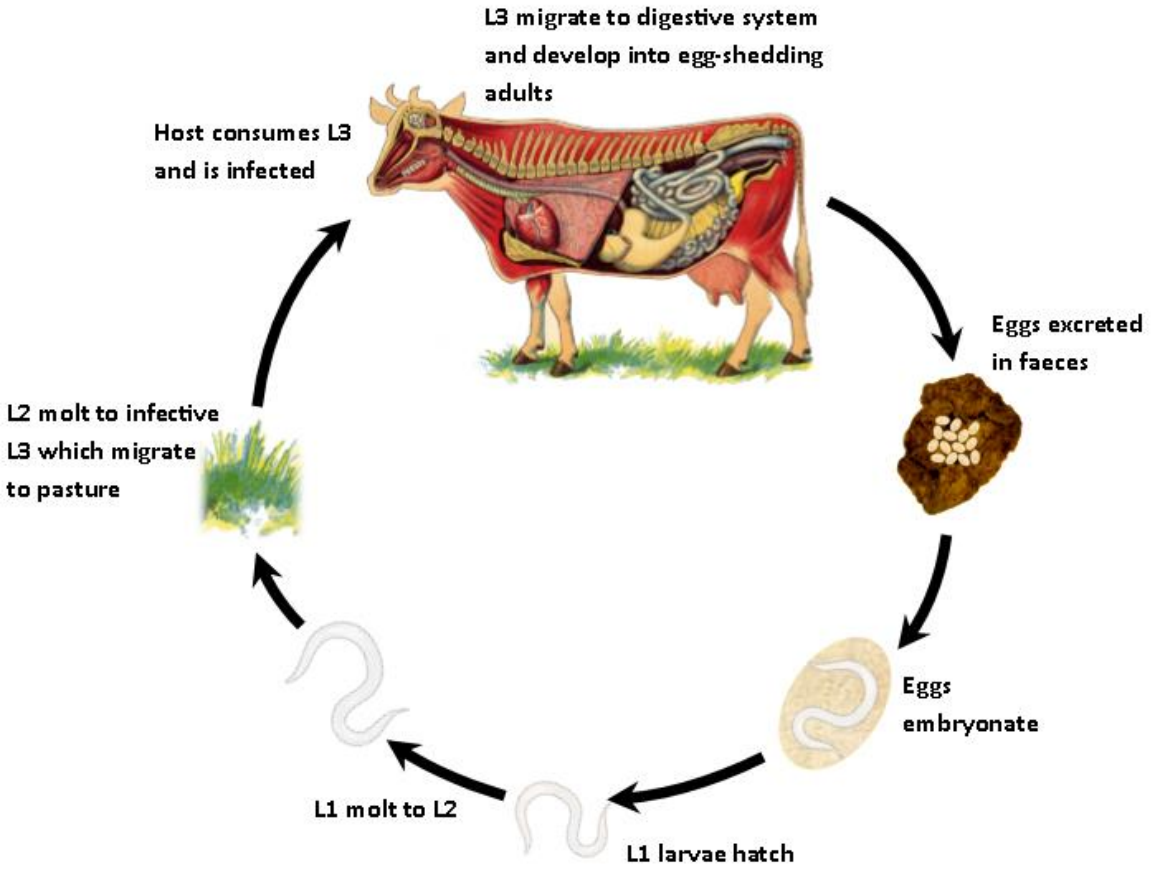


Figure 1.2 - The typical lifecycle of gastrointestinal nematodes (GINs) in cattle. Source: original, using image of cattle edited from Buffum (1905).

All of these factors and their impacts are driven by livestock management practices. Effective livestock management is no small feat, and there is certainly no unique right or wrong strategy. Management requires the consideration and balancing of the whole range of agricultural factors, within the context of a system's own goals and ethos. Effective management has to be informed and, therefore, requires a system-level understanding of livestock production, founded on scientific research and evidence.

1.1.4 UK beef production

The UK's beef industry produces approximately 900,000 tonnes of beef per year (FAO, 2017), the majority of which is produced by pasture-based grazing systems. Total production equates to produce worth around £2.8 billion annually (AHDB, 2017), with substantial indirect value added elsewhere across the food production industry and the wider economy. Over the past half-century, the UK beef industry has been through a period of significant change, facilitated by major advances in the scientific understanding of animal production and by technological advances. This has led to an increase in animal productivity from a mean carcass dressed weight of approximately 265kg 1980 to over 350kg in 2016 (FAO, 2017). Simultaneously there has been a decrease in the total head of cattle. However, these two factors have balanced out, and total meat production has remained relatively static, despite a significant increase in the UK's human population over that period (FAO, 2017). Increases in animal productivity have been fundamental to improving resource efficiency within grazing beef systems. The increase in individual animal productivity, as driven by breeding, nutrition, and management, is biologically and ethically limited and may be beginning to plateau (Figure 1.3). In the developed world, between 1962 and 2006, mean carcass weight increased 64% (165kg to 271kg). However, it is predicted that from 2006 to 2050 this value will only increase by 4% (to 283kg) (FAO, 2006). Such a plateau would represent a halt in the industry's primary driver (increases in live weight) of efficiency increase. This may create a shift in focus towards meat quality and system sustainability (both environmentally and economically), both of which hold the potential to increase profitability in the long-term. Given the substantial size of the industry and its role in the UK economy and society, the sustainability and improvement of the industry are of high importance.

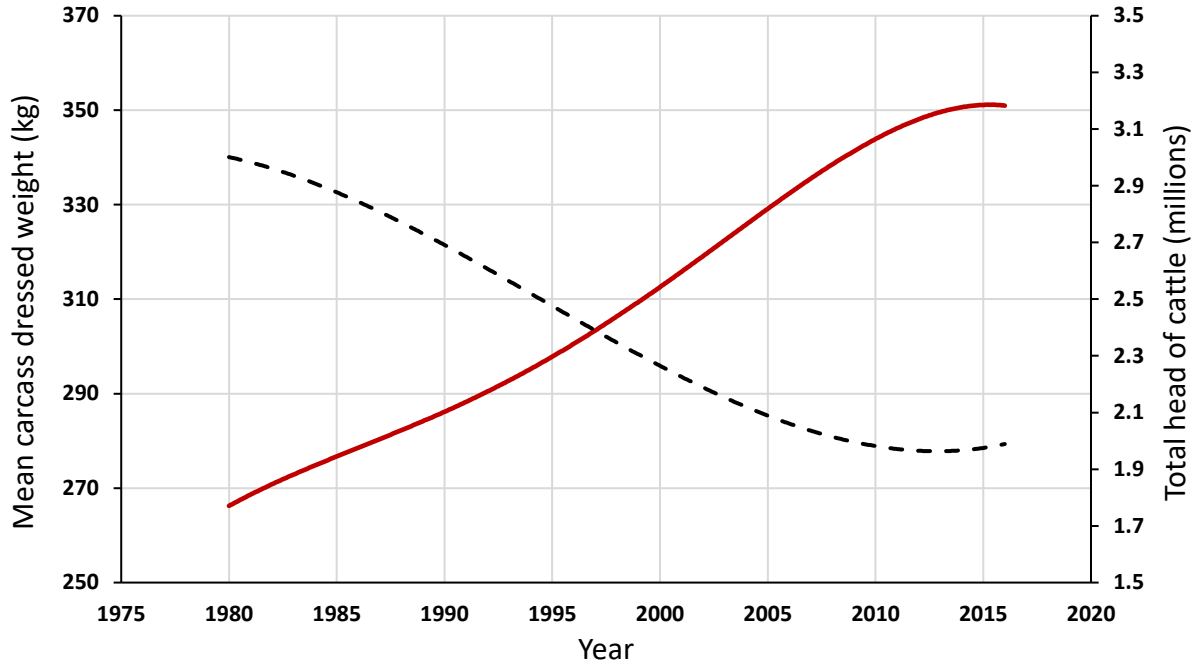


Figure 1.3 - Mean dressed weight and total head of cattle within the UK from 1980 to 2016 (FAO, 2017).

The rapid and significant productivity increases that have been seen in the past are unlikely to continue into the future. One reason for this is the realisation that high increases in short-term productivity can be detrimental to animal and system health. As a result, there needs to be a push towards ensuring long-term sustainability, as opposed to simply creating high yields. Another major reason is that the progress over recent decades has been brought about through the application of fundamental science, such as an improved understand of genetics, enabling rapid progress at a rate which is not sustainable. As this continues, advancement will come from more specific and high-tech research, often focussing on seemingly small factors. It is the cumulative effect of optimising smaller aspects of the system that will yield significant overall benefits. Achieving this requires scientific research that does not just focus on one factor or use one approach, but instead, draws together different factors using different methodologies. By combining approaches such as computer modelling, controlled studies, and case studies, it is possible to develop a contextual and rounded view of the different aspects of beef production and identify gaps in our understanding.

1.2 Management

The overwhelming majority of beef produced in the UK comes from pasture-grazing cattle, a stark contrast to feed-lot systems which are commonplace throughout other parts of the

world. Grazing systems are far more complex and dynamic than feed-lot systems and, consequently, there are more potential options for variations in management and efficiency improvements. There is no precise 'best practice' for grazing cattle systems and how a farm is managed and operates is dependent on a range of complex and interacting factors. One example is climate, which varies significantly across the UK with both latitude and longitude. Northern regions are typically colder than Southern, while Western regions are typically wetter than Eastern. This has wide implications across the system and can become a limiting factor to production and productivity. For example, fresh-water mud snails are the intermediate host of the parasitic liver fluke (*Fasciola hepatica*). Therefore, liver fluke prevalence is highly linked with weather (Fox et al., 2011; Ollerenshaw, 1958). Therefore, farms in such regions must consider that as part of their management strategy, this could involve changes in grazing and the use of veterinary intervention.

1.2.1 Rotational grazing

Pasture-based beef production systems often practice rotational grazing, moving cattle between different fields/areas throughout the grazing season. Successfully implemented rotational systems allow for pasture to have fallow resting periods over which flora can regenerate and regrow, improving pasture productivity, reducing the need for external inputs, and therefore increasing farm carrying capacity. Furthermore, rotational grazing has been successfully implemented for the control of parasitic diseases by 'breaking' their lifecycles. Although widely used in conventional farming, these potential benefits are of great appeal to organic farms.

1.2.1.1 Pasture productivity

Chapman et al. (2003) conducted a controlled experiment comparing set-stocked (where animals remain on one field for a prolonged period of many months) and rotational grazing systems under both high and low fertiliser inputs. Results showed that rotational grazing led to greater total herbage production, and therefore supported a greater stocking density. The study also found that rotational management also significantly impacted pasture composition, with the rotational systems yielding a tonne less of clover (dry matter basis). As a consequence, the major growth benefit was to the primary grass crop. It was concluded that either management style, in isolation, would mean missing out on the positives of the others

and that an integrated and varied management system has the potential to harness the positives of both. This is somewhat concurrent with prior observations by (Brougham, 1960) who found that changes in grazing management throughout the year could be a tool for improving pasture. For example, intensive grazing during the autumn would reduce the abundance of dominant summer species; this would decrease short-term yields as the conditions would not allow the summer species to re-establish, however, this would allow for winter species to establish more easily. The primary findings of Chapman et al. (2003) are mirrored across many other studies. Stobbs (1969) examined rotation grazing of Zebu cattle in Africa, finding that it yielded benefits when herbage was low, and suggesting it promotes pasture recovery. This study also found significant differences in pasture composition between rotational grazing and set stocking systems. Walton et al. (1981) conducted a controlled experiment which looked in more detail, including pasture productivity concerning animal weight gain per hectare, which is a better real-world measure for beef production systems. It was found that cattle weight gain was 218 kg/ha in the rotational system, compared to 119 kg/ha in their continuously grazed equivalent, the increase due to the ability for greater stocking densities. It was also found that that the percentage of alfalfa in the sward increased from 23 to 47% under rotational grazing and that animals graze for 2.4hrs less per day. The rotational system yielded more digestible forage with higher proportions of protein, calcium, magnesium, and copper. These examples highlight the importance of pasture management and trade-offs involved in pasture management and how, ultimately, it is down to an informed preference on a farm-by-farm basis. Farms that practice this most effectively will be more profitable and economically sustainable.

1.2.1.2 Parasite control

Gastrointestinal nematode (GIN) eggs, deposited in faeces, are not immediately infective, L1 larvae must hatch from the eggs and moult twice before becoming infective L3. The length of time this takes depends on the species and weather. For example in high humidity and warm temperatures (20-25°C) *Haemonchus contortus* and *Trichostrongylus colubriformis* eggs can become infective L3 in as little as 3-4 days (Hsu and Levine, 1977; Veglia, 1916). Whilst at 10°C this process may take 16 days (Smith, 1990), or longer if conditions are dry. It is thought that by moving animals onto fresh pasture before deposited eggs become infective again, that the parasite lifecycle can be 'broken' or at least that pasture is not grazed when it is most

infective. This means that the peak infectivity of a parasite population, on pasture, is avoided by available host animals. After this peak numbers steadily decline and, therefore, the longer that pasture is left fallow, the lower the risk of infection to livestock when they return to graze that pasture (Stromberg and Averbeck, 1999). A number of studies have found rotational grazing effective for the control of parasitic diseases of grazing ruminants (Barger et al., 1994; Larsson et al., 2006; Marley et al., 2007; Stromberg and Averbeck, 1999), although in some instances no significant benefit has been observed (Eysker et al., 1993; Kunkel and Murphy, 1988). The effectiveness of such strategies is inevitably dependent on factors such as the nematode species present and the local weather and climate, which may explain why some studies have found it to be effective and others not. It is possible that an ineffective rotational grazing system might even increase parasitic infections at a herd level. Rotational grazing typically requires high stocking densities (with respect to the area of land immediately being grazed), therefore if a rotation were slightly slower than parasite development, i.e. if parasites became infective within five days, but rotations were weekly, cattle would be exposed to an unusually high density of infective larvae.

1.2.2 Organic farming

The UK organic food industry is growing steadily, and currently, approximately 15% of cattle in the UK are classified as organic (DEFRA, 2017). There are various definitions of 'organic', with respect to food production. Perhaps the most widely used is that of the Soil Association, who set out a comprehensive set of standards which they use to certify farms. The standards aim for the '*highest possible standards of animal welfare, environmental and wildlife protection*" (Soil Association, 2017a). In many aspects, these standards are significantly more stringent than those required by law. For beef production, such standards include that cattle must be free range for >200 days per year, no artificial fertilisers are to be used on pasture, a minimum 60% grass-based diet, no antibiotics to be used prophylactically, and that pesticides are limited in use. For many farmers, these standards fit in with their own ethos. However, it is important to consider that livestock production is a business and that additional regulations can constrain profitability.

There is a range of pros and cons to organic farming, further complicating the balance which management must achieve. A key downside is typically a loss in productivity, in beef

production systems this is often characterised by a reduced stocking density (Stockdale et al., 2001) and carcass weight (Woodward and Fernández, 1999), resulting in a higher production cost of meat (Fernández and Woodward, 1999). This reduced gross performance productivity is not always directly reflected by a lower economic productivity as it is somewhat mitigated by the potential benefits, such as reduced inputs and increased meat value.

1.2.2.1 Environmental impact

Organic systems have been shown to reduce the dependence on external farm inputs due to improved soil fertility and biodiversity, providing a cost saving and evidence for system sustainability (Bengtsson et al., 2005; Maeder et al., 2002; Stockdale et al., 2001). A key feature of organic farms is that they do not routinely use synthetic fertilisers, herbicides, or pesticides, all of which have environmental costs due to their production and impacts (Figure 1.4). Therefore, the removal of these components has the potential to reduce the environmental impact of production, so long as yields are not disproportionately diminished as a result.

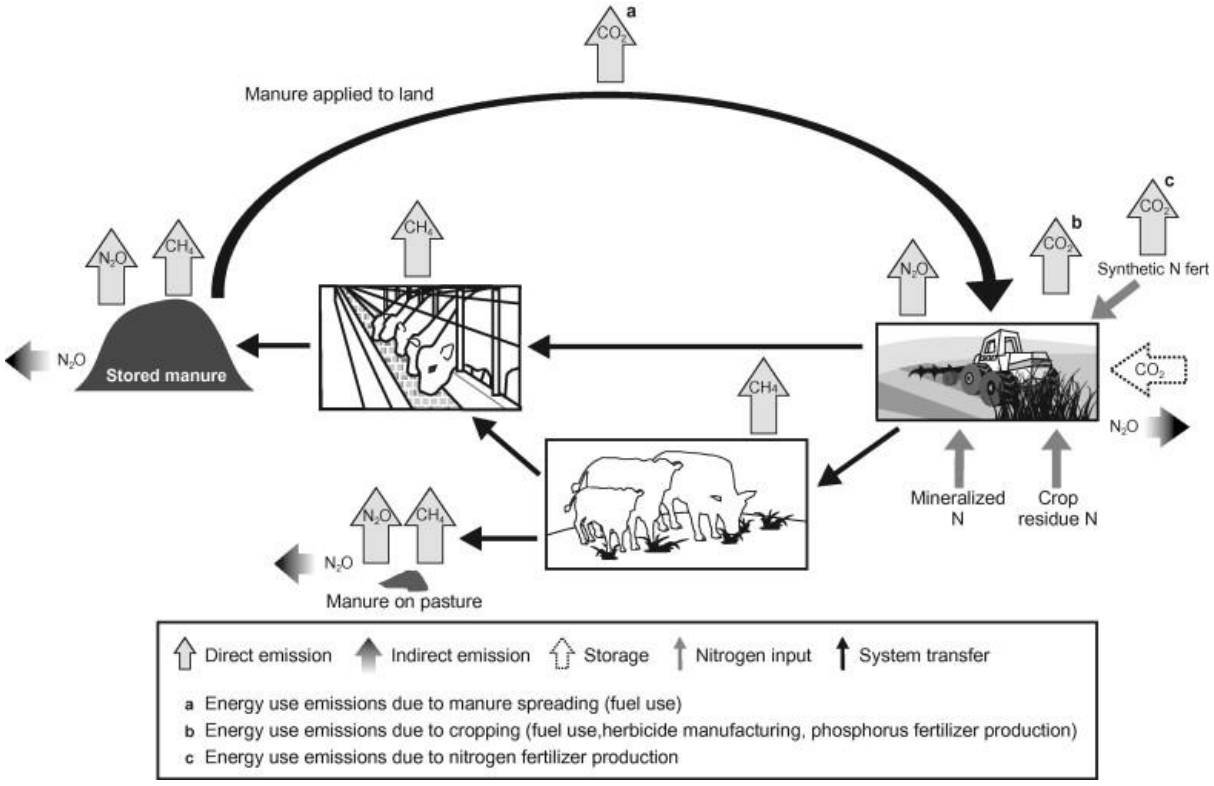


Figure 1.4 - Inputs and emission sources of conventional beef production. Whilst components labelled "a" are relevant to both conventional and organic farming, all components labelled "c" and some labelled "b" are not a part of organic systems. Source: Beauchemin et al. (2010).

Flessa et al. (2002) compared conventional and organic beef production and found that organic systems produced lower greenhouse gas emissions per hectare of land, however when adjusted for a reduced beef yield, systems did not differ. The study did not account for land use efficiency, doing so could provide evidence that the conventional system has (net) lower greenhouse gas emissions than the organic system. For example, the land freed up through the more intensive conventional system could be converted to woodland, which would reduce the net greenhouse gas emissions over the same area and sequester carbon. A significant study in this field, by Tuomisto et al. (2012), conducted a meta-analysis of the environmental impact of organic farming (arable and livestock), compared to conventional systems. The results of Flessa et al. (2002) were mirrored in this study, finding that the total negative environmental impact per area was reduced, but that this was not necessarily reflected relative to yield.

1.2.2.2 Carcass characteristics

Carcass characteristics have been shown to differ between organic, intensive, and conventional systems. Woodward and Fernández (1999) found that conventionally reared steers had a significantly higher carcass weight than organic steers, along with larger rib eyes (a value cut) and less fat – which is not necessarily considered positive or negative, as fats can dictate taste. These factors may be somewhat linked to the higher weight gain and feed conversion (irrespective of dry matter intake) when compared to organic animals (Fernández and Woodward, 1999). However, it is notable that Blanco-Penedo et al. (2012) found that, despite similar disease incidence, organic cattle carcasses were significantly less condemned at abattoir, implying a health advantage while also partially mitigating for loses due to decreased carcass weight, characteristic of organic beef production.

1.2.2.3 Ensuring benefits

There are potential benefits to the organic production of beef. However, these can easily be overshadowed by reductions in yield. Therefore, if achieved, improvement of yield on organic beef farms towards the levels achieved by conventional systems would provide a strong justification for organic beef production. Two main objectives need to be achieved to accomplish this; the first is to ensure animal productivity and the second is to ensure pasture productivity. Animal productivity is primarily achieved through ensuring that animals are free from disease and have high-quality nutrition. Pasture productivity can be achieved by

ensuring soil health and providing flora with optimal conditions for growth. Effective management of rotational grazing has some potential in addressing both of these, through reducing the risk of parasitic infections and by enhancing soil and pasture health and productivity, as previously outlined.

1.3 Gastrointestinal nematodes and cattle

Parasitic diseases are a primary source of losses within beef and livestock production systems worldwide. In particular, GINs can cause substantial losses due to their impact on animal health and performance, resulting from underlying pathology. GIN lifecycles are relatively simple when compared to cestodes or trematodes which have intermediate hosts such as mites and snails. However, this simplicity has allowed them to become ubiquitous throughout the world in both wild and domesticated mammals, resulting in significant productivity losses in livestock. Therefore, the prevention and control of GINs is a key consideration for improving the environmental and economic sustainability of beef production systems.

While the diagnosis of parasitic diseases can be relatively straightforward through the use of faecal egg counts (FEC), which are widely practised by veterinarians, farmers, and researchers, there is scope for greatly improving the tools that are available for diagnosis and for developing new tools. This could facilitate more precise and effective treatment of parasitic diseases within a targeted selective treatment (TST) programme. Improving the management of treatment has the short-term benefits of improving individual animal health, but also the long-term benefit of mitigating the development of anthelmintic resistance in helminth populations (van Wyk, 2001; van Wyk et al., 2006). Such resistance occurs when anthelmintics place a selection pressure on parasite populations, favouring the inheritance of genes which enable individuals to tolerate the drugs. The more broadly this pressure is applied to a population, the more likely it is that resistant phenotypes prevail. In addition to the impact of parasites on animal health, it is also necessary to consider the environmental impact of parasitic diseases, caused by anthelmintic usage, and the potential wider impacts of these on farm productivity and performance.

1.3.1 Prevalence and impact

1.3.1.1 Animal health and pathology

A key pathological sign of GIN infections is a reduction in feed intake. Feed intake studies can be conducted by either (or both) artificially introducing an infection to observe with a control group of non-infected animals, or by introducing an intervention measure with a control group of infected animals. Experimental examples of both options have yielded analogous findings. Forbes et al. (2000) found that cattle that had not been treated with anthelmintics (ivermectin) grazed for an average of 105 minutes per day less than their treated counterparts, resulting in 0.78kg less feed intake, similar results were observed by Bell et al. (1988). Taylor et al. (1989) found that artificially infected animals had reduced feed intakes, but that there was a threshold - calves trickle infected with 10,000 *Ostertagia ostertagi* larvae daily had a reduced feed intake, whereas those infected with 2000 daily did not. The exact mechanism that causes this behaviour is not fully understood.

Gastrin, a hormone that stimulates gastric acid secretion, has long been associated with GIN infections. Within ruminants, the gross effect of gastrin is a reduction in reticular contraction and abomasal emptying, both of which result in a slowing of the passage of feed through the rumen. Grovum (1981) found that levels impacted feed intake in sheep. Work led by Professor Mark Fox (Royal Veterinary College, University of London) explored the role of gastrin as a part of the mechanism for feed intake suppression. In response to GIN infections, finding that elevated gastrin levels reduced food intake of cattle (Fox et al., 1989a, b, c; Fox, 1997; Fox et al., 2002). Similarly, Fox et al. (2006) observed that lectin, a hormone which regulates energy expenditure through controlling appetite, may be linked to reduced food intake in response to GIN infections.

A compounding factor to reduced food intake is feed conversion rate (animal weight gain per weight of feed consumed), which can be reduced due to GIN infections. Goldberg (1965) found that artificially infected animals had a reduced feed conversion of 9.2-12.2 percentage points. Comparable results were also found by studies investigating the positive impacts of anthelmintic treatment on feed conversion (Bauck et al., 1989; Leland et al., 1980; Williams et al., 1991). Part of the reasoning for this is the cost to the host of mounting an immune response to infection.

The culmination of these combined factors is a total reduction in animal weight. Borges et al. (2013) observed that GIN infection levels, as measured by FECs, negatively correlated with calf weight. Analogous findings have been presented by Ploeger and Kloosterman (1993) and Devaney et al. (1992), whom both looked specifically at calf weight gain. Devaney et al. (1992) reported a difference in weight of 10 kg, over 16-20 weeks, between uninfected and infected animals. Similarly, (Dimander et al., 2000, 2003) observed that low-level GIN infections of calves could cause losses of 30-60 kg within their first 12 months, whereas Sutherland and Leathwick (2011) observed 14 kg of loses over the same period. Such gross productivity losses have an impact on final sale weight and therefore the total profit per animal.

1.3.1.2 Economics

Economic losses, to livestock producers, from GIN infections, are caused by a range of direct and indirect factors. The most prominent measure is animal performance (weight maintenance and gain), a factor driven by the underlying pathology of infection.

Further direct losses occur through the use of anthelmintics and the cost of veterinary services. Anthelmintics are used to simultaneously improve animal health and profitability. Therefore, there is an economic argument for their use as a money saving device. Leland et al. (1980) found that that the feed efficiency benefits at 28 and 51 days, yielded by anthelmintics, outweighed the cost of treatment. Across the EU (European Union), annual sales of anthelmintics are estimated to equate to €400 million (Selzer, 2009). The ability to lessen the use of anthelmintics, through informed management strategies, would yield economic benefits to livestock producers as well as a health benefit to the animals. Examples of this are the use of targeted selective treatment and selective breeding for diseases resistance (Malan et al., 2001; van Wyk et al., 2006).

Estimating the economic impact of any particular diseases is incredibly difficult due to the diversity of the direct and indirect losses involved (Morgan et al., 2013; Sutherland and Leathwick, 2011). The impact of GIN diseases can be yet more complicated to estimate as, in cattle, they do not always cause notable pathology meaning that they may not be treated or even diagnosed. In such instances, small inconspicuous productivity loses may occur. While no figure can be realistically calculated, there is evidence that provides a picture as to the economic scale of the problem. A 2002 report by DEFRA (DEFRA, 2002) estimated the cost of

a range of livestock diseases. The 15 target diseases of cattle they examined cost approximately £402 million (£610 million when adjusted for inflation for 2016 (Bank of England, 2017)) to the industry. Of those, two helminth diseases were included, parasitic bronchitis and fasciolosis, costing £9.5 million and £23 million respectively.

Estimations of the economic impact of animal diseases are calculated on an annual basis. This, therefore, does not account for the long-term impact of current behaviours. A prime example of this is the increasing prevalence of resistance to veterinary medicines due to their current usage (Sutherland and Leathwick, 2011; Teuber, 2001; Waller, 1994, 1997), the potential future financial impact of this could be tremendous, conservatively in the hundreds of millions of pounds. Given all of the information available, it is reasonable to believe that GIN infections of cattle annually cost the UK beef producers tens of millions of pounds with the cost of all helminth diseases in the hundreds of millions or more.

1.3.2 Diagnostics

A range of diagnostic methods are available for the identification of GINS, each with specific benefits and drawbacks. Faecal egg counts (FEC) are the most commonly used methods and, while they have significant merit, provided limited information on the true impact of infection on the host. There is also significant variation between FEC techniques, therefore limiting the external validity of results (Bosco et al., 2014; Levecke et al., 2012; Rossanigo and Gruner, 1991). A range of molecular methods are technically feasible, such as polymerase chain reaction (PCR) diagnostics (Zarlenga and Higgins, 2001) and enzyme-linked immunosorbent assays (ELISAs) (Keus et al., 1981). However, these are not widely used or well-established within veterinary science, outside of an academic context. Improving the convenience and resource requirements of immunological methods, as has been achieved within human medicine, would promote the uptake of such methods. Molecular methods are necessary for the accurate identification of nematode species (McKeand, 1999) and would help to inform control strategies at local and national levels. Combining different diagnostic approaches has the potential to provide comprehensive bodies of evidence to inform treatment decision making. For example, FECs could be used to identify parasite species, but also to identify individual hosts that may require further health assessment by immunological and molecular methods.

1.3.2.1 Immunological diagnosis

The parasitic trematode, liver fluke (*Fasciola hepatica*), attracts significant scientific attention. While it is not as ubiquitous as GINs, where it does strike (the west of the UK), its impact can be devastating. Enzyme-linked immunosorbent assays (ELISA) are commercially available for detection of *F. hepatica* in cattle (Farrel et al., 1981) and sheep (Zimmerman et al., 1982), by identifying specific antigens in sera, plasma, and milk. ELISA can identify infection as soon as six weeks post-ingestion, and the assay requires an incubation time of about 1hr 45mins. Also, the method is highly sensitive. The main drawback of ELISA is the cost of the kits, making diagnosis notably more costly than FEC. Due to the requirements of the test, it is not currently possible for farmers to conduct testing themselves. However, research by Gordon et al. (2012) has shown great promise for a coproantigen ELISA that utilised faecal samples, further development and success could lead to more efficient and practical detection of *F. hepatica* within livestock worldwide. Similarly, ELISAs have been utilised for the detection of other parasitic diseases, Cornelissen et al. (1997) used the method for the detection of bovine lungworm (*Dictyocaulus vivparus*). A prime practical example of this application is the detection of carbohydrate larval surface antigens (CarLA) in sheep saliva (Merlin et al., 2017).

Within human medicine, faeces (or "stool") is routinely used for the diagnosis of disease, through the quantification of immunomarkers. A small number of studies have applied this principle to veterinary medicine, successfully using faecal markers to detect specific diseases or assess animal immunology (Duménigo et al., 1996; Peters et al., 2004; Watt et al., 2015; Wedrychowicz et al., 1985). Nevertheless, such uses of faeces have received little attention, yet hold great potential to enhance veterinary diagnostics. The development of effective faecal diagnostic tools would reduce the need for time-consuming and welfare-negative invasive procedures while possibly providing novel information on gastrointestinal health.

Human medicine has seen significant innovation in the development of immunological diagnosis methods, leading to a number of cheap, rapid, and effective, molecular diagnostic tools that can be utilised in the field. One example is the circulating cathodic antigen (CCA) dipstick (Figure 1.5), a lateral flow device which can detect *Schistosoma* spp. antigens in host urine (Sousa-Figueiredo et al., 2013) and are now routinely used. The technology is founded on the same principles of ELISAs, a solution or product with the target molecule moves laterally along the device until it reaches an indicator strip which captures and reacts to the

target molecule, indicating its presence. Such levels innovation has not been seen in veterinary medicine and represent a key area in which major advancements can be made. The technology and scientific capabilities exist to make such advancements.

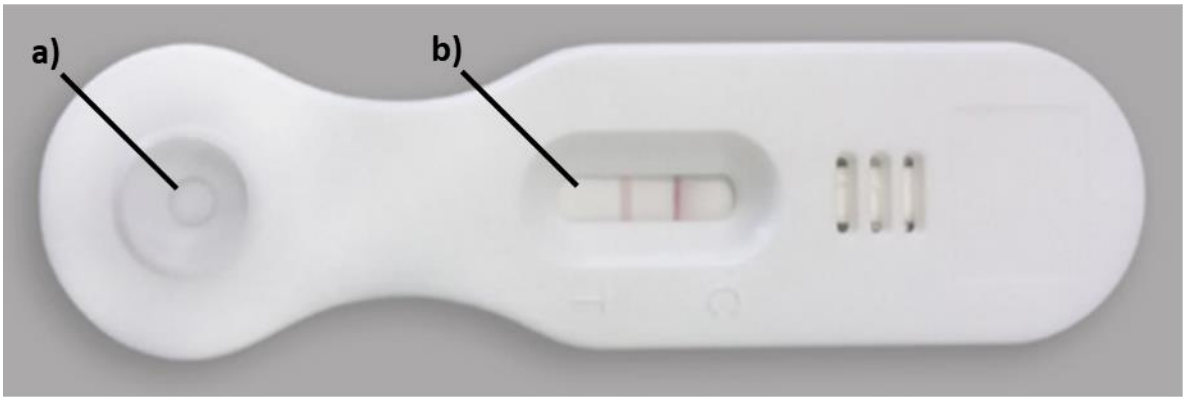


Figure 1.5 - Circulating cathodic antigen dipstick (CCA). A practical tool used for the diagnosis of schistosomiasis in humans, through detecting antigens of Schistosoma *spp. in urine. a) Receiver, where sample is applied b) test-strip, indicating if sample if positive. A similar tool could potentially be created for rapid diagnostic of gastrointestinal nematodes (GINs) and other agriculturally significant parasites. Source: Rapid Diagnostics (2015).*

1.3.2.2 Faecal egg counts

Faecal egg count techniques typically rely on the isolation and separation of parasite eggs from faecal material, followed by microscopic identification and manual counting of eggs. The eggs per gram (epg) of faeces is then calculated and used as a proxy measure the severity/intensity of infection. A wide variety of techniques are available, each with their own benefits and shortcomings (Bosco et al., 2014; Levecke et al., 2012). The primary benefits of FEC techniques are that they are usually quantitative, cheap, and simple to conduct. This means that they can be used worldwide irrespective of resource limitations. FECs, however, are by no means perfect. The spatial overdispersal of eggs within faecal material is an issue impacting most FEC techniques, leading to large sample variability (Carstensen et al., 2013; Lester et al., 2012). Mature parasites shed eggs in cycles and therefore repeat sampling of the same individual can yield significantly different results within a short period. This mechanism can also facilitate spatial clumping of eggs within faeces, meaning that sub-samples may not be representative. Furthermore, the water content of the faeces has the potential to dilute or concentrate eggs, resulting in biased egg counts (Le Jambre et al., 2007). This is particularly important considering that diarrhoea is symptomatic of many gastrointestinal parasitic infections and drought events can lead to outbreaks of parasitic diseases. All of these factors

can compound, culminating in inadvertent biases. Levecke et al. (2012) compared standardised FLOTAC, McMaster, and Cornell-Wisconsin FEC techniques in the context of drug efficacy testing and found that all of the methods produced bias. Furthermore, these biases were not consistent across the techniques nor were they consistent for the same techniques tested at different egg concentrations.

1.3.3 Anthelmintic treatment

Anthelmintics are the primary class of drug used to combat helminth infections of livestock. Their use is widespread and routine, not just as a reactive treatment for diagnosed infections, but also prophylactically. The ubiquitous administration of anthelmintics has been one of the driving forces of increased animal performance, by reducing animal energy expenditure on immune responses and by preventing damage to organs. However, this has not been without cost, resistance to anthelmintics is becoming increasingly prevalent and poses a significant risk to livestock production worldwide (Borges et al., 2013; Köhler, 2001; Papadopoulos, 2008; Papadopoulos et al., 2012; Sutherland and Leathwick, 2011; Waller, 1994, 1997). The development of anthelmintic resistance, due to overuse and inappropriate use, means that the industry will be less able to react to specific outbreaks as and when they occur. It also means that animals suffering the most may not be able to benefit from effective medication. Furthermore, the use of anthelmintics has an environmental impact due to the non-target insecticidal properties of anthelmintics of all classes (Beynon, 2012; Beynon et al., 2015; Strong, 1993; Strong and James, 1993; Wall and Beynon, 2012). While this all may sound particularly negative, there is an important place for anthelmintics within veterinary medicine. Anthelmintics need to be used more sparingly and not as a first resort; this will increase the longevity of their efficacy.

Arguably, the largest issue regarding parasite control is sustainability. Resistance to anthelmintics is a constant hurdle to treatment, and it is, therefore, vital to conserve the efficacy of current drugs for as long as possible. For sustainable parasite control, we must focus on prevention rather than treatment and combine multiple control methods, with different targets, to do this, thus altering farming practice as a whole. Such approaches include, but are not exclusive to anthelmintic use, grazing rotations, nutrition, selective breeding, control of movement, and climate monitoring. Analogous to the theory of

combination drug therapy, combining different control methods into one strategy greatly reduces the likelihood of an individual having a resistance genotype to the entire strategy, compared to its singular components.

1.3.3.1 Anthelmintic resistance

The resistance of helminths to anthelmintic medication represents a major threat to UK and worldwide livestock production. Reports from across the world have identified resistance to every major anthelmintic, on every content (Borges et al., 2013; Papadopoulos, 2008; Papadopoulos et al., 2012; Sutherland and Leathwick, 2011; Waller, 1994, 1997). The consequence of anthelmintic resistance is the inability to be able to effectively respond to significant infections or outbreaks, with the potential of major loses.

Although scientific literature on the topic is readily available, anthelmintic resistance has received relatively little public attention when compared to antimicrobial resistance (AMR), which in recent years, has taken centre stage in medical and agricultural politics within the UK. This has led to positive changes across those industries, catalysed by significant media coverage of AMR and commentary from influential individuals. This is down to a host of social and scientific factors. Bacterial diseases are far more prevalent in developed societies than helmintic diseases are. As a result, the general public has a far greater awareness and personal investment in antibiotics than they do anthelmintics. The majority of individuals in the UK will have taken antibiotics at numerous points in their life, while they most likely will have never taken anthelmintics. Anthelmintic resistance in the developed world is also not, directly, an anthropogenic issue, it is a step or two detached from the day to day lives of most. The more rapid reproductive cycle and the ubiquity of pathogenic bacteria, compared to helminths, does make AMR a more imminent and serious threat. However, anthelmintic resistance is likely to become an increasingly significant issue if actions are delayed until it is too big to ignore.

Reducing the use of anthelmintics and the prevalence of resistance necessitates more precise and informed practice across veterinary medicine. There is also the need for farmers to implement non-medicinal control strategies to prevent disease, these include rotational grazing management, high levels of nutrition, adequate biosecurity measures, and hygienic operation.

1.3.3.2 Environmental impact

The environmental impact of anthelmintics is well-studied and the chemical mechanisms well understood. Anthelmintics, administered to cattle, are not fully metabolised or degraded and, consequently, are excreted by the animal in dung and urine (Gover and Strong, 1995; Iglesias et al., 2006; Sommer et al., 1992). When these residues enter the ecosystem, they exert non-target effects on invertebrates and other organisms, reducing their populations and impacting local ecology (Adler et al., 2016; Barth et al., 1993; Beynon, 2012; Floate, 1998a; Gover and Strong, 1995; Iglesias et al., 2006; Madsen et al., 1990a; Sommer et al., 1992; Sommer and Bibby, 2002; Sommer and Nielsen, 1992; Wall and Beynon, 2012). The mechanism for this effect varies between anthelmintic classes. For example, macrocycling lactones (e.g. ivermectin) bind to glutamate-gated chloride channels of invertebrate nerve and muscle cells, permanently opening them resulting in paralysis and ultimate death (Cheeseman et al., 2001; Köhler, 2001; McCavera et al., 2009; Njue and Prichard, 2004; Wolstenholme and Rogers, 2005).The impact of this effect extends beyond that of biodiversity, as the impacted species play vital roles in the degradation of dung and nutrient cycling, all of which impacts soil quality and associated factors (Barth et al., 1993; Floate, 1998a; Wall and Beynon, 2012). When combined with the potential negative impact of anthelmintic resistance, this evidence supports lessening the use of anthelmintics.

There is somewhat of a stagnation of research into these impacts, with large similarities between studies that take the approach of comparing the activity of an invertebrate(s) in dung with or without anthelmintic residues. A key factor that has not been touched on is how the applications of anthelmintics are managed at a herd level, which would provide more representative information on the non-target impacts of anthelmintics within the real world. In recent years the implementation of TST programs has changed how anthelmintics are administered, which is likely to have affected the way anthelmintic residues interact with dung fauna. Part of the reason for the lack of research into this specific aspect may be the scale and complexity of implementing such an experiment. Therefore, computer modelling may be a prime candidate to simplify the system and minimise hurdles such as ethics and resource availability. This has been attempted once already by Boxall et al. (2007). However, the model was fundamentally flawed due to an inadvertent cap that the algorithm places on

anthelmintic toxicity. Nevertheless, the work was important in highlighting the issue and laying a basic framework that could be utilised in the future.

1.3.3.3 Targeted selective treatment

Targeted selective treatment is an approach to the use of veterinary medicines which uses diagnostic individual animal information to decide which individuals require treatment. Significant evidence for the benefit of TST has led to it becoming the recommended 'best practice' for the treatment of livestock against parasitic diseases. TST has been found to lessen the selection pressures that favoured anthelmintic resistance genes, therefore, slowing the rate by which resistance genes become prevelant. By not treating the entire herd refugia is formed, and a proportion of non-anthelmintic-resistant parasites survive, slowing the rate at which resistant genes become prevalent (Figure 1.6) (Kenyon et al., 2009; van Wyk, 2001; van Wyk et al., 2006). A refugium of dung without anthelmintic residues is also simultaneously created for invertebrates (Cooke et al., 2017).

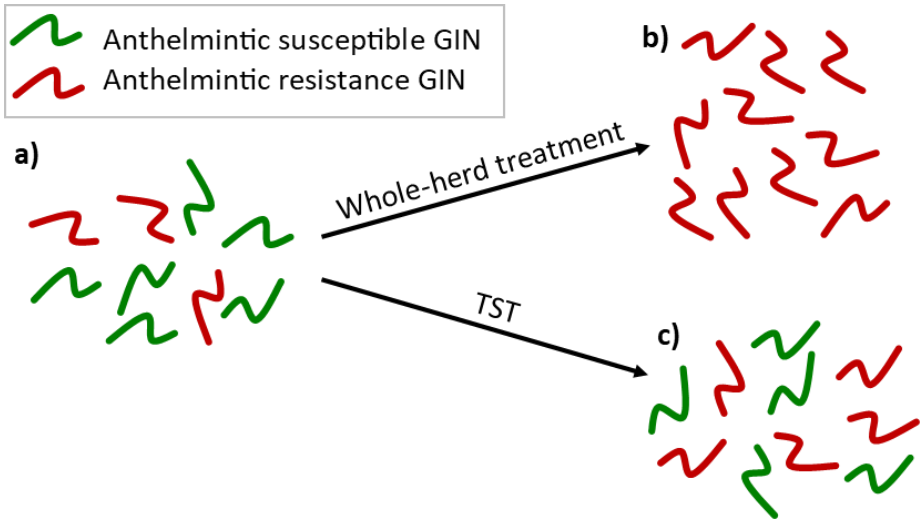


Figure 1.6 - Mechanism for refugia formation through targeted selective treatment (TST) strategies. a) original gastrointestinal nematode (GIN) population with some resistant individuals b) parasite population after whole-herd anthelmintic treatment, with all GINs resistance c) population after TST, with an increased number of resistant individuals, but susceptible individuals still present. Source: original.

A key aspect of TST is an effective health assessment which is used to inform treatment. Depending on treatment goals, diagnosis of an infection is not necessarily enough information to warrant treatment. Vercruysse and Claerebout (2001) proposed two thresholds for treatment. The first is a 'production-based threshold'; this is a sub-clinical level at which the

cost of treatment is outweighed by its benefits, particularly with regards to ensuring weight gain and future infection. The higher threshold is the “therapeutic threshold”, the level at which immediate treatment is necessary for animal health. In many systems these two thresholds will not be the same, however, the closer they are brought together, the more beneficial a treatment regime can be to both animal health and economic productivity. The formal definition of such thresholds is also not always feasible and therefore may be a subjective threshold to which objective information is applied to. In order to assess an animal in relation to these thresholds, it is necessary for there to be a range of effective diagnostic tools. This is an area where significant advancement can be made. Bath and van Wyk (2009) developed a five-point checklist (for small ruminants) for informing TST treatments, representing a significant advancement by defining a clear procedure. The checklist looked at gross health markers, eye colour, body condition, tail cleanliness, jaw shape, nasal discharge, and coat condition. These are useful metrics for assessing general health, especially as an initial assessment, but provided limited information as to the cause of infirmity and internal pathology.

1.4 Conclusion

Significant advances in UK beef production have enabled the industry to respond to challenges over the past three decades. In addition to the historical and persistent challenges, such as consumer demands for low prices and pressure to reduce environmental impacts, new challenges are emerging. The most pertinent of these is maintaining the rate of industry advancement in response to emerging challenges. There is no one key area in which improvements are necessary, and instead, widespread continual and incremental advances are necessary. This requires a multidisciplinary scientific approach to assess numerous factors, utilising numerous approaches, in relation to one-another. No particular farming system/approach is intrinsically superior to another. In particular, the literature surrounding organic farming and rotational grazing show that the strategies can yield benefits or costs, depending on how they are implemented. Successful implication of farm management strategies requires farms to be informed about their systems, animals, and the implications of their decisions. This can be most widely achieved through the development and application of scientific research, investigating the multivariate factors of farming systems. In support of that, solutions need to be practical for real-world use by farmers, veterinarians, and other

stakeholders. Achieving all of this is the best strategy to tackle the challenges of pasture-based beef production within the UK and worldwide. Furthermore, it can find and develop effective strategies to improve the balance between productivity and environmental impact in the short and long-term.

The objective of this thesis is to test the overarching hypothesis that intentional management of dung as a critical resource on-farm has multiple benefits that improve the resilience of beef production systems. The hypothesis is tested using a multi-disciplinary approach and gathers evidence from a range of sources, from individual animal data through to farm-level case studies.

Chapter 2

Dung dynamics - Biochemical composition and degradation of cattle dung from three typical UK grazing systems.

Summary

A key consideration in the management of pasture-based livestock systems is pasture type. Acting as the primary source of nutrition for livestock, forage type and quality have the potential to be major drivers of gross farm productivity. However, the importance of sward management is not only derived from its impact on animal development but due to a wider role in nutrient cycling and local ecology. Three typical UK grazing systems were compared to investigate differences in the individual characteristics of each system's forage and dung. In addition, the manner by which dung degrades within each system was measured. Significant differences were found in the composition of forage and dung, as determined by a range of biochemical and physical metrics. Furthermore, the rates at which dung degraded, both regarding total organic matter and specific biochemical components, varied significantly between systems. Variations in dung degradation rate were found to be 69% driven by pasture type on which the dung degraded on and 4% by composition of the dung. Results provide strong evidence to show that pasture type alone has the potential to significantly impact the nutritional properties of cattle dung, with a knock-on impact on important ecological and environmental processes. When combined with other management decisions, such as the use of anthelmintics, these differences could compound on a large scale to great effect.

2.1 Introduction

Dung is a vital agriculture resource that is involved in the recycling of nutrients from livestock to the soil, facilitating a host of complex and interactive biological process relevant to system productivity and the wider environment (Figure 2.1). The rate and manner in which dung degrades may influence the nature of nutrient turnover and incorporation by impacting upon factors such as leaching potential and digestibility of the dung by invertebrates. There is, therefore, a necessity to further understand the drivers and dynamics of dung degradation. In addition, artificially applied fertilisers, both organic and inorganic, are applied sporadically in bulk, whereas the deposition of cattle dung via defecation is a much more continuous and consistent input. Consistent inputs are more stable and resilient whereas bulk applications of fertilisers may increase the susceptibility of nutrient run-off, leading to reduced nutrient-soil incorporation and increasing the potential for negative environmental impacts such as eutrophication (Hart et al., 2004; Smith et al., 2001). This may occur, for example, if large quantities of fertilisers were applied shortly before a period of heavy rainfall.

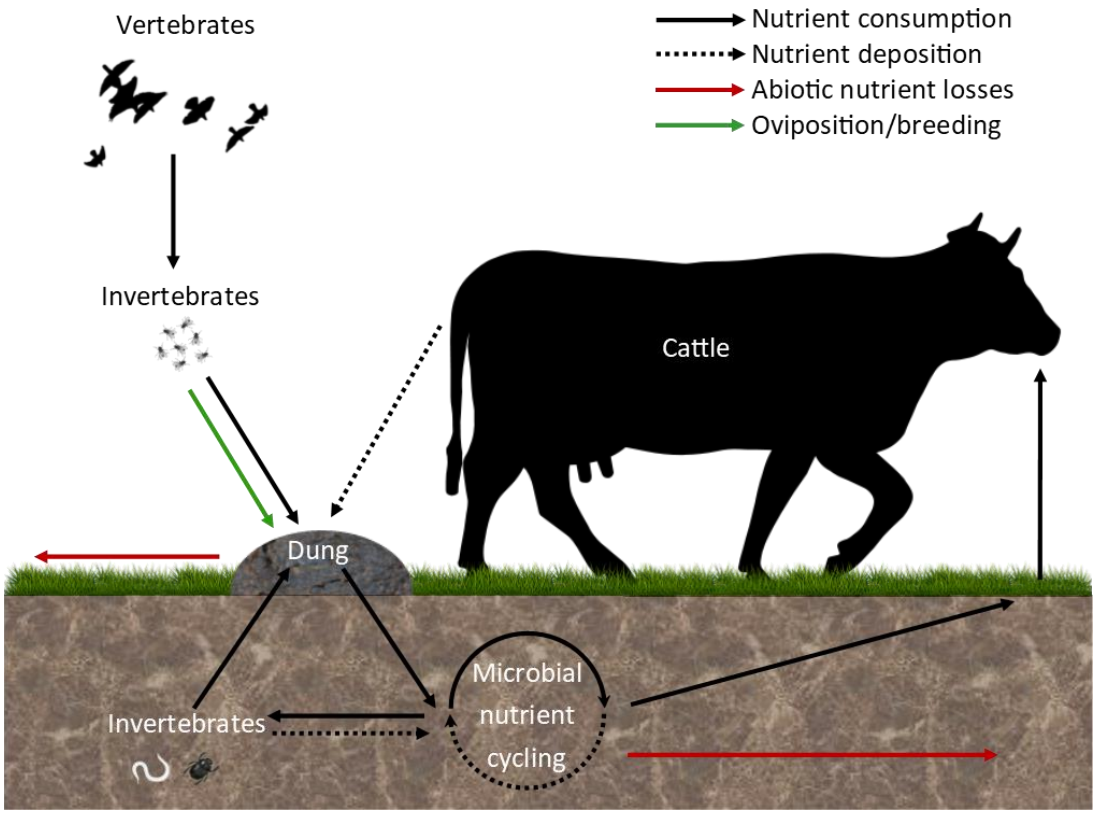


Figure 2.1 - Diagram showing the basis of the dung cycle within grazing livestock systems. Source: original.

Dung is also a central component in the lifecycle of gastrointestinal nematodes (GINs) and other significant parasites, which rely on dung as an environment for their transmission onto pasture and for the hatching of eggs and development of larvae. Whilst little is known about the impact of dung dynamics on the transmission of GINs, it is feasible to consider that factors such as composition and degradation may influence the development and transmission of such pathogens.

Grazing livestock systems are incredibly complex and with that complexity comes a host of management decisions which must be made. One of the primary considerations is pasture type. In these systems, pasture is central to the network of factors and variables which farm management must consider on a regular basis. Pasture management, therefore, is instrumental in driving factors such as soil quality, forage quality, biodiversity, hydrology, and most crucially, animal health. This impact upon biology has the potential to subsequently impact the characteristics of waste products from the animal, especially dung. If pasture type were to significantly impact the composition of cattle dung, it is feasible that this may alter the rate and manner in which dung degrades, with knock-on impacts throughout the dung cycle and wider farm system.

2.1.1 Dung as fertiliser

A significant focus is put upon the characteristics of artificially applied fertilisers, and great consideration is taken to optimise nutrient levels of these fertilisers before application. This is done to optimise yields but particularly important as such fertilisers are introducing new nutrients to the system. The Agriculture and Horticulture Development Board (AHDB) have produced the Nutrient Management Guide, which provides practical information on the typical nutrient contents of fertilisers and their application (AHDB, 2010). This reflects the importance of fertilisers within agriculture systems and the need to optimise their uses to enhance yield and reduce their environmental impacts. Despite the detailed attention that fertilisers receive, there is significantly less active consideration as to the composition of dung/manure, which is applied artificially to pasture or deposited naturally by cattle. Cattle dung is a valuable resource in pasture-fed beef production systems and is a key component of the nutrient cycle (W. F. Sheldrick et al., 2003). Dung, returned to pasture, facilitates the turnover and recycling of nutrients from the animal, back into the soil, where they become

readily available for soil biological processes. (Aarons et al., 2009; Ayoola and Makinde, 2008; During et al., 1973). A typical adult animal within a beef herd can produce upwards of 7 tonnes of dung in a typical 170 day grazing season (Lorimor et al., 2004). When upscaled to herd level this represents a significant amount, and therefore even small nutritional differences in dung composition could manifest as a large difference in gross nutrient inputs between different systems. Furthermore, there are estimated to be over 1.5 billion cattle in the world (FAO, 2017), a number which is set to increase above 2 billion by the year 2050 (FAO, 2006). Any change, no matter how small, can have a significant net impact globally. These differences could impact a range of other biological processes, in the environment, leading to variations in soil quality, pasture productivity, and in forage nutritional quality, which is pertinent to animal health and performance. This represents a key gap in our knowledge of livestock production systems.

Dungait et al. (2005) investigated the dynamics of nitrogen, carbon, and phosphorus within livestock systems (Figure 2.2), showing the complex and diverse interactions that occur throughout grazing systems. This would become exponentially more complex with every added element, whether it be the inclusion of more nutrients, biodiversity, or environmental factors, highlighting the sheer complexity of agricultural systems.

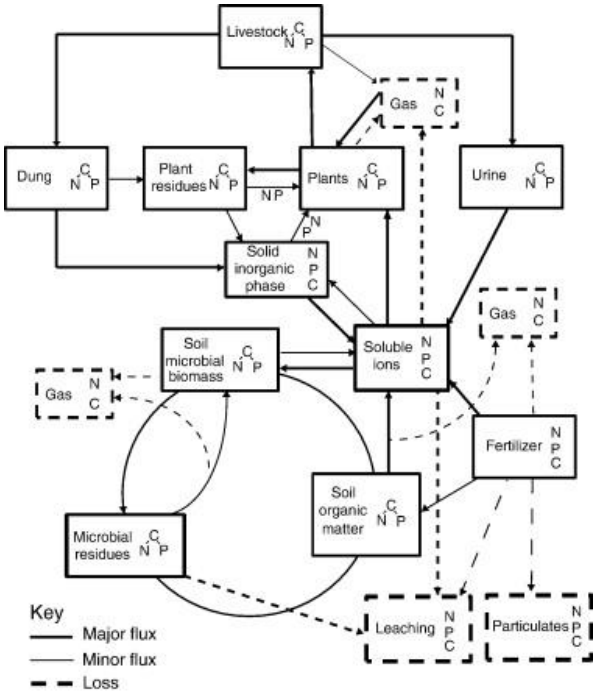


Figure 2.2 - Model of the complex interactions of the N, P and C cycles in a mixed farming (arable crops and livestock) system. Covalent bonding between elements is indicated by C—N—P. Source: Dungait et al. (2012).

2.1.1.1 Legislation and regulation

There have been significant attempts to control the use of fertilisers in an attempt to mitigate for their potential environmental impact. The Nitrates Directive, set out by the European Commission (1991), aims to protect water systems from agriculturally derived nitrates. As part of this, nitrate-vulnerable zones (NVZs), were defined across the UK, in which the application of nitrogen fertilisers and the storage of organic manures must be controlled in accordance with specific regulation. A key focus is placed upon the amount of nitrogen that can be applied annually per hectare, relative to yield. For manures, the cap is 170kg of N per hectare for conventional manures and 250kg for organic manures. While this focusses purely on nitrates, water systems are further protected by the Water Framework Directive (European Commission, 2000) and subsequent Ground Water Directive (European Commission, 2016), which are much broader 'catch-all' directives that cover additional aspects such as phosphates, particulate, and fertilisers run-off. These directives highlight the environmental importance of understanding and controlling dung dynamics. Furthermore, the regulations that they impose have the potential to influence farm management. It is therefore important that the tools and understanding are available to enable farms to utilise dung as a resource for maximum benefit, within regulation. Effectively achieving could yield benefits to farms and the wider environment.

2.1.2 Epidemiology

Dung also plays a vital role in the transmission of numerous agriculturally significant pathogens. As outlined in Chapter 1 (1.3), GINs, along with other helminths, rely on the passage of eggs through dung. The dung provides a relatively stable environment for embryonation, development, and survival. Bryan and Kerr (1989) reported that cattle dung that persisted through a drought led the accumulation of GINs in dung on the pasture. During rainfall these GINs migrated out of dung en masse, leading to a 10-fold increase in GIN larval pasture contamination. It is clear that this event was caused by the inhibition of dung degradation. There is significant evidence showing that insect activity can reduce parasite transmission through the consumption and burying of eggs and that other organisms such as bacteria, viruses, fungi, and predatory nematodes also feed on free-living stages of parasitic nematodes (Fincher, 1973; Larsen et al., 1994; Larsen, 2000; Waller, 2006; Waller and Faedo, 1996). Many of these species, such as dung beetles, are also positively associated with the

degradation of dung and recycling of nutrients. Furthermore, other pathogens can be transmitted through cattle dung. Arguably the most topical of these is *Mycobacterium bovis*, the bacteria that cause tuberculosis (TB). Whilst *M. bovis* can persist in cattle faeces, the degradation of dung by invertebrates may reduce or remove the risk of transmission (Duffield and Young, 1985; Phillips et al., 2003). Therefore, it is feasible to consider that rapid degradation of dung, facilitated by invertebrate activity, may also be beneficial for the control of pathogenic diseases of livestock.

2.1.3 Biodiversity

Mature grazing cattle predominantly obtain nutrients through the consumption of pasture forages. However, a portion of these nutrients is not retained by the animal and is instead excreted in dung, thus returning them to pasture and wider environment. The turnover of these nutrients is facilitated by soil microbiology, with soil microbial biomass being an effective marker of soil health and fertility (Rice et al., 1996; Schloter et al., 2003). Lovell and Jarvis (1996) found that application and beef cattle dung to soil significantly increased soil microbial biomass and respiration, both positive indicators of soil health and fertility. Analogous findings have been found around the world in a variety of agricultural systems (Belay et al., 2001; Ghoshal and Singh, 1995; Rochette and Gregorich, 1998; Witter et al., 1993). Rochette and Gregorich (1998) found that this effect was cumulative. In turn, soil microbial biomass is also driven by forage and sward structure, particularly rhizosphere characteristics (Haynes and Francis, 1993). The consequence of increased soil health and fertility is a potential reduction in dependence on external fertiliser inputs, increased pasture productivity, and increase system biomass and biodiversity. All of these factors may manifest as improved profitability and a reduced negative environmental impact. From this example it is clear to see the cyclic nature of nutrients within grazing livestock systems, further highlighting the significance that variations in dung and pasture composition play in system health.

In addition to its agricultural importance, dung has value as a source of nutrients within the wider environment by acting as a food source and refugia for invertebrates and other organisms (Marshall, 1977; Standen, 1984). These, in turn, can help to facilitate nutrient cycling while also enhancing local ecology, biodiversity, and system health (D'arcy-Burt and

Blackshaw, 1991; McCracken et al., 1995). Equally, agricultural fertilisers, including manures, have the potential to harm the wider environment. A prime example of this is the nonpoint pollution of surface waters, which can lead to eutrophication of water systems and subsequent reductions in wildlife communities (Correll, 1998; Ulén et al., 2007). However such impacts of agriculture can be mitigated by reducing excess nutrients within the systems (Carpenter et al., 1998), reaffirming the need to more comprehensively understand the nutrient makeup of dung. Given the vital role of cattle feed and dung, in order to optimise the efficiency of beef cattle production, it is crucial that we understand the characteristics of feed and the dung which it subsequently produces.

2.1.4 Forage composition

Forages are routinely analysed for their nutritional composition. Indeed, animal nutrition is a heavily studied subject, and as a result, a large industry has emerged from it. It is common for farmers to have forages, especially silage, analysed for nutritional composition, with the goal of improving forage quality and animal growth. Goering and Van Soest were pioneers in this field with many of today's modern methods and definitions based on the techniques that they developed. Many companies now offer commerical forage analysis services, for around £10 per sample for near-infrared spectroscopy (NIRS) analysis. Macronutrients (i.e. protein, fats, carbohydrates, and fibre) drive bulk metabolic processes such as digestion and growth, while micronutrients (elements, such as magnesium and iron) play much more subtle roles on a molecular level. Imbalances in either can have significant impacts on animal health and performance. However, such analysis is predominantly limited to forages, with limited consideration of other materials, particularly dungs and manures, for which the methods are transferable.

During winter, cattle are typically housed and fed on silage, grass that has been tightly packed in air-tight bales and left to ferment. After ensiling, bales are left for a period of weeks to ferment. During this time, differences in the starting properties of bales (such as moisture, pH, and oxygen) can manifest as significant differences in silage quality and composition at the time of consumption (Muck, 1988). As a result of the fermentation process, complete silage analysis requires investigation of additional factors such as pH and lactic acid content, which are not typically considered for fresh forage (Charmley, 2001). Filya (2004) compared

fresh maize to 90-day ensiled maize and observed higher concentrations of crude protein and organic matter but lower concentrations of fibrous components, showing the impact of ensiling forages on their composition.

2.1.4.1 Macronutrients

As a primary part of forage analysis, the biochemical composition of feeds is operationally defined by various groups of compounds gravimetrically (Figure 2.3). While exact methods vary, underlying principles and groups are consistent. Traditionally, forage analysis is conducting using wet chemistry techniques, pioneered by Goering and Soest (1970), however rapid and nondestructive techniques, such as near-infrared spectroscopy (NIRS), are becoming increasingly common (Batten, 1998). Although commonly called 'forage analysis', the techniques are not exclusively for utilisation on forages and are used widely across the food industry. There is also the potential for the techniques to be applied to agricultural materials, such as dung and manures (Batten, 1998; Burns and Ciurczak, 2001; Stitcher et al., 1969).

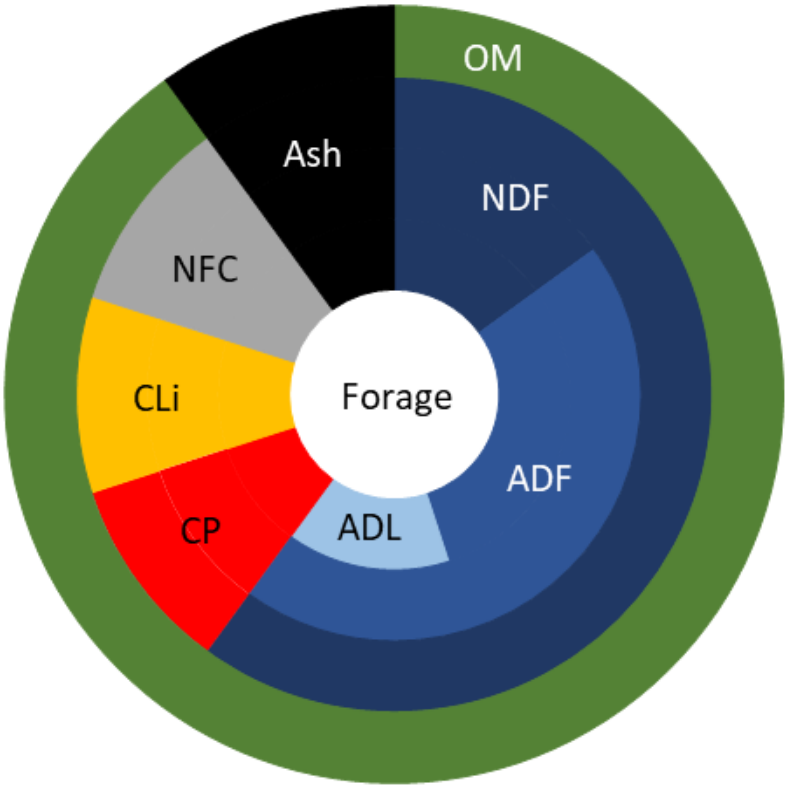


Figure 2.3 - Multi-level pie chart showing typical components of forage analysis of dried material and their respective sub-components. Inner levels are sub-portions of outer levels. Proportions are arbitrary and not to scale. OM = organic matter, NDF = neutral detergent fibre, ADF = acid detergent fibre, ADL = acid detergent lignin, CP = crude protein, CL = crude lipids, NFC = non-fibre carbohydrates.

Organic matter (OM) is a fundamental measurement when analysing agricultural materials such as feeds, dungs, and soils. However, with regards to forage, it provides only the most basic information as to the 'quality' of a feed and the components of OM need to be measured independently. OM is typically measured by loss on ignition. Anything not classified as OM is considered as ash.

Neutral detergent fibre (NDF) is the most commonly analysed component of forage and is often used as a proxy for forage digestibility (Mertens and Ely, 1979; Oba and Allen, 1999), with higher NDF suggesting higher quality forage. The NDF portion of forage is comprised of cellulose, hemicellulose, and lignin, all of which are plant cell wall constituents. While cellulose is more complex than hemicellulose; both are relatively simple when compared to lignin, which is a highly complex molecule whose primary function is to provide strength and rigidity to plant structure. These varying structures have a significant influence on their digestibility, with lignin being highly indigestible. Arelovich et al. (2008) found that, in beef cattle, small increases in NDF intake increased net energy intake, without compromising feed efficiency. NDF is traditionally quantified through wet chemistry, by the digestion of forage in a pH neutral detergent, which strips away proteins, lipids, and carbohydrates (Goering and Soest, 1970).

Acid detergent fibre (ADF) is a component of NDF comprised of cellulose and lignin. The process for determining ADF is similar to that of NDF, however, also removes hemicellulose (Goering and Soest, 1970). ADF is highly indigestible for ruminants, and therefore ADF is inversely related to forage digestibility. ADF is typically quantified similarly to NDF, however, with an acid detergent, capable of dissolving hemicellulose through the hydrolysis of hemicellulose chains, breaking them into smaller monomeric sugars which acid can degrade (Binder and Raines, 2010).

Acid detergent lignin (ADL) is furthermore a sub-component of ADF and is only comprised of lignin. Lignin reduces feed digestibility and is often used as a predictor of such. While fibre is considered a valuable component of feeds, the ADL sub-component is not. Therefore, a lower proportion of ADL, compared to ADF, is generally sought after. ADL is determined by acid soaking of ADF, to remove cellulose, followed by ashing to determine lignin (Goering and Soest, 1970).

Crude protein (CP), despite only making up around 10-15% of cattle dry matter intake, is a vitally important component, often used as part of quality assessment (Mertens and Ely, 1979). High protein feeds (such a soybean, linseed, and other grains) are often used as supplements to beef cattle (Byers and Moxon, 1980; Galyean, 1996). Protein is a high-value resource and facilitates cattle growth and feed efficiency, with respect to carcass weight amd price. CP increases dry matter (DM) and NDF digestion, contributing to body weight maintenance and body condition, these effects are especially pertinent during times of stress, such as winter calving (Beaty et al., 1994).

Crude lipids (CL) represent the insoluble fats of forage and typically makes up the smallest proportion of forage when compared to other components. This is due to the predominantly grass-based diet of pasture fed livestock. In feedlot systems CL content of feed may be higher, for example, cotton seed contains as much as 175g kg^{-1} (dry matter basis) of CL, compared to concentrations of around 100g kg^{-1} in many pasture forages (National Research Council, 2000).While all dietary components are important; crude lipids are generally not considered to be of central importance. CL is typically quantified by solvent extraction (in which lipids are soluble) and filtration through cellulose membranes (Nielsen, 2003; Thiex et al., 2003).

Non-fibre carbohydrates (NFC) are calculated as the remainder once the other components have been accounted for. However, that is not to mean that it is any less important. NFC is highly digestible and therefore a valuable energy source, in addition, this energy promotes microbial growth and digestibility within the rumen (Hoover and Stokes, 1991). NFC has the potential to impact NDF digestibility, both positively and negatively, and it is the ration of NFC to NDF that is important. However, that ratio is dependent on rumen pH and the effect of NFC on pH (Arroquy et al., 2005; Haddad and Grant, 2000).

The book "*Nutrient Requirements of Beef Cattle*" (National Research Council, 2000) includes a library of feeds which has information on typical nutrient values of many common forages. A selection of those feeds are outlined above, for reference (Table 2.1).

Table 2.1 - Typical nutrient values of various common livestock feeds (National Research Council, 2000).

	Nutrient (g kg^{-1} DM)				
Feed	**NDF**	**Lignin**	**CP**	**Fat**	**Ash**
Alfalfa silage (full bloom)	510	120	160	27	80
Clover (ladino hay)	360	670	224	27	94
Legume pasture (spring)	330	26	280	27	100
Wheat (straw)	789	130	35	20	77
Barley (silage)	568	31	119	29	83
Maize cobs (ground)	870	68	28	6	18
Maize silage (40% grain)	450	40	92	31	40
Barley malt	460	30	281	14	70

2.1.4.2 Micronutrients

In addition to bulk increase in liveweight, nutrients play much more subtle roles in animal health. Deficiencies and overabundance of particular nutrients can have significant health implications. For example, deficiencies in selenium increase susceptibility of cattle to disease (Boyne and Arthur, 1981; Koller et al., 1983; Stabel et al., 1989), while an overexposure can be toxic (selenosis) (Koller and Exon, 1986; Olson, 1986). Similarly, deficiencies in protein and zinc can increase susceptibility to GINs by inhibition of antibody production (Coop and Holmes, 1996; Coop and Kyriazakis, 1999; Scott and Koski, 2000). Balancing micronutrients can, therefore, be difficult without supplementation (Spears, 1995), bringing about the possibility of systemic losses through reduced animal performance, costs of reacting to infection, and costs of preventative measures. 'Hidden hunger' is a form of malnutrition in which micronutrient deficiencies lead to pathogenesis despite there being a seemingly adequate supply of consumable feed. While typically associated with humans, livestock are also susceptible to the condition. Many of the symptoms of nutrient deficiencies are similar,

such as wasting, lethargy, and anaemia. As a result, the diagnosis and treatment of micronutrient deficiencies can be difficult.

Micronutrient deficiencies are common worldwide, but perhaps most impactful in developing nations where the resources are not always available to provide rounded diets or to respond to incidence. These are also the regions in which livestock are most depended on for milk, meat, and strength. A farms susceptibility to micronutrient deficiencies is dependent on a wide range of factors, such as soil and pasture properties (Gissel-Nielsen et al., 1984, 1984; Gupta et al., 2008).

2.1.5 Research objectives

The rate at which dung degrades is important for many processes relating to animal and environmental health. In this chapter, we used the North Wyke Farm Platform to test the overarching hypothesis that: Cattle diet drives the composition of cattle dung and its subsequent degradation in grassland systems and that improved forage management may provide options to control and improve system health. We tested this using four sub-hypotheses:

Hypothesis 1 – The composition of cattle dung, from the three studied grazing systems, differ significantly.

Hypothesis 2 – The composition of feed (fresh herbage and silage), from the three studied grazing systems, differ significantly.

Hypothesis 3 – The rate of degradation of fresh dung varies significantly based upon the composition of that dung and the grazing system in which that dung is.

Hypothesis 4 – The gross mass of dung pats, from the three studied grazing systems, will be significantly different.

2.2 Methods

Dung, herbage, and silage were taken from three typical UK farming systems. The dung was initially analysed to quantify various chemical and physical properties. Representative samples of dung were then taken from each of the three systems and used in a dung degradation experiment where the biochemical properties of dung were analysed over a time

series. All three of the studied systems were represented at the study site, Rothamsted Research's North Wyke Farm Platform (Devon, UK; Hatch et al., 2011; Orr et al., 2016). Each system is represented by an individual 'farmlet', an independent and isolated system functioning within the larger farm. Each farmlet has its own independent herd of beef cattle and at the time of sampling each herd was predominantly made up of Charolais crosses, and British Blue crosses. Other than the variation in pasture, the farmlets are similar. The site (50.76950,-3.90128; Appendix 8.3) was chosen to reduce the impact of variation in uncontrollable variables, such as weather, climate, and topography, and also due to the high amount of data available for the site as a whole. The three grazing systems that were represented in the study are as follows:

Increased legumes – These plots have a high proportion of white clover (approx. 30%) sewn into the pasture. The nitrogen-fixing properties of the legumes mean that the increased legume fields have a reduced fertiliser input. This is known as the blue farmlet (B).

Permanent pasture **–** Enhanced by inorganic fertilisers, this ley has been in place for at least 20 years on each field within that system. This is known as the green farmlet (G).

High sugar monoculture **–** At the time of the study, under a high sugar monoculture, these fields are reseeded every five years with whatever is considered as the most novel and suitable new grass variety at the time. This is known as the red farmlet (R).

2.2.1 Dung and feed collection

On each farmlet, silage is produced annually from herbage derived from the farmlet's own supply and fed only to cattle from that farmlet. At the time at which it was fed to cattle (14/01/15), ten grab samples of silage (approximately 300g dry weight) were taken and pooled for each farmlet. Herbage samples were collected (22/07/15) along a 13 point W-transect across one field within each farmlet; herbage was cut at approximately 4cm from the ground to represent grazing height (Grant et al., 1996) and combined to form a composite sample. All samples were stored in tied plastic bags within clip-top plastic drums at 4°C for no longer than two days before being prepared for analysis.

Dung samples were taken from the cattle twice. Initially, when they were being fed on a silage diet (14/01/15)and again when they were grazing on pasture (22/07/15). It was ensured that

cattle were on their specified diet for at least one month before sampling, thus excluding the significant influence of past diet on dung composition (Dungait et al., 2005). During the first sampling, fresh dung samples were collected by waiting for the individual to defacate and recovering dung from the ground, being careful not to incorporate any foreign material. Dung was thoroughly mixed and transferred to 150ml, screw top, plastic containers.

During the second sampling, more dung was required to analyse dung degradation rates. In this instance, > 18 kg of dung was collected from each farmlet. Dung was collected by monitoring cattle, immediately after defecation their dung was collected into plastic bags using a clean ladle, bags were then cable tied and stored in slurry containers at 4˚C for no longer than two days.

2.2.2 Dung degradation

An 84-day dung degradation field study was conducted to analyse the rate of dung degradation of different dungs across the different grazing systems and to investigate how the composition of cattle dung changes over time. The field trial began on 24/07/15 and ran for 84 days until 16/09/15. After 28, 56, and 84 days four dung pats of each type were randomly selected to be removed from each field plot. Cattle were turned out between 14/04/15 and 24/04/15. Weather data for the experimental period is available in Appendix 8.1.

Collected dung was homogenised using a clean and dry cement mixer. This formed a representative and distinct dung type from each farmlet, named b, g, and r, respectively. Five-hundred grams (wet weight) portions of dung were then dispensed into pre-weighed plastic bags. A sub-sample of each dung type was retained for analysis. On each farmlet (B, G, R), artificial dung pats of each type (b, g, r) were placed. Thus nine groups were formed (Bb, Bg, Br, Gb, Gg, Gr, Rb, Rg, Rr).

Dung pats were formed by placing a Ø15 cm plastic cylinder on the centre of the mesh and squeezing dung out of their bags into the cylinders. Bags were retained and reweighed to determine the weight of any residue. Thus the weight of each dung pat was known. Pats were placed directly on 20x20 cm green plastic garden mesh, of a 20 mm aperture, held down in the corners by 9-inch steel pegs. Each farmlet had an individual field plot (Figure 2.4), which measured 5 x 5 m surrounded by an electric fence to prevent disturbance from livestock. No

faeces or other notable materials and features (such as trees, rabbit holes etc.) were present inside or within 10m of the plots. The outer 55cm within each plot was unused and left as a buffer strip. The experimental area inside of the buffer zone was split into a 6 x 6 grid of 36 subplots, each measuring 65 x 65 cm. Dung pats were placed in the centre of each plot, leaving a 50 cm gap between pats to prevent mixing and direct invertebrate migration. Pat location within field plots was designated by a controlled random block design, using a random number generator, with the caveats that each row and column contained two pats of each dung type and that each quadrant contained three pats of each dung type (Appendix 8.2). The location of field plots was chosen for topographic similarity, all with North-West (45˚) slopes and a rise < 0.5 m (Appendix 8.3).

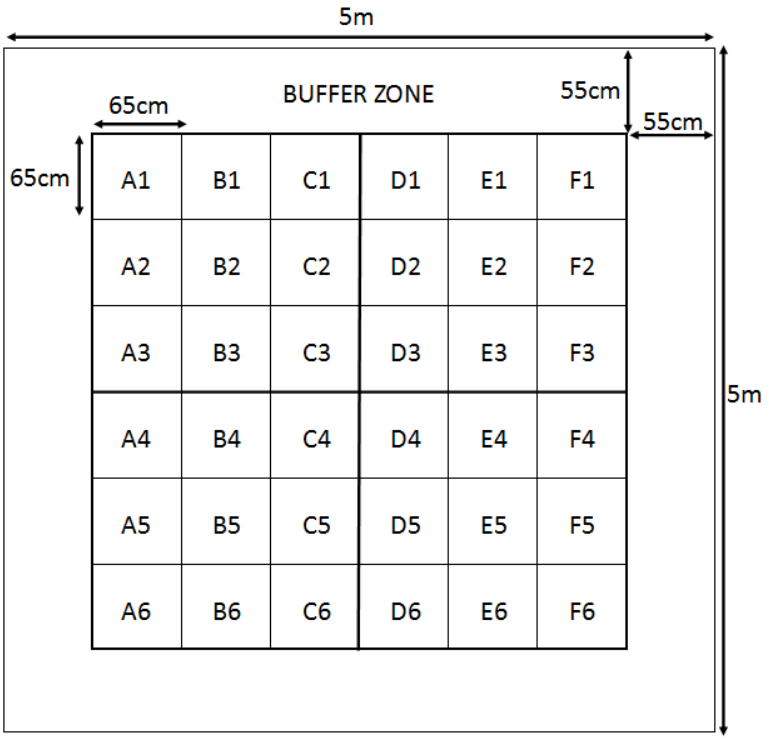


Figure 2.4 - Schematic of plot design for placement of artificial dung pats on each of the three farmlets. Each dung pat had a unique 65 x 65 cm square in the 6 x 6 grid. The grid was protected by a 55cm buffer zone to ensure it was not disturbed by animals.

2.2.3 Dung and feed analysis

The collected dung, herbage, and silage (including that from the degradation experiment) underwent an array of analyses to quantify specific compounds and physical characteristics. Unless otherwise stated, all materials underwent all analyses.

2.2.3.1 Sample preparation

Samples were prepared for analysis by being oven dried at 65°C until a constant weight and ground to < 2 mm in an electronic grinder (Bosco BCG01) with stainless steel blades. Processed samples were stored in screw top plastic containers at 4°C. Weights, taken during this process, also gave results on the proportion of moisture and dry matter in samples.

2.2.3.2 Organic matter (OM) and ash

OM and ash were determined by loss on ignition at 360°C to a constant weight (> 6 hrs). Porcelain crucibles were washed, pre-furnaced, and allowed to cool in a desiccator. Then, 0.5 g of dried and ground sample was weighed into the crucible, before furnacing. OM was determined as the loss in weight of the sample and crucible, as a proportion of the original sample weight. The remainder was classified as ash.

2.2.3.3 Neutral detergent fibre (NDF)

Neutral detergent fibre was determined by crucible methods, in accordance with EN ISO 16472, using cold and hot extraction units (FOSS: FT 121 Fibertec™ and Fibertec™ 8000, respectively). Firstly, 0.5 g of sample was added to sintered crucibles (porosity 2) along with 0.5 g of sodium sulphite (to prevent protein clumping). Crucibles were fixed into place in the cold extraction unit and soaked in acetone while agitated by pressurised air. After five minutes the acetone was aspirated off, removing the majority of non-polar lipids. This acetone rinsing process was repeated thrice.

Crucibles were then secured in the hot extraction unit and underwent a digestion process in neutral detergent solution (NDS) (Ankom FND20C) with *n*-octanol as an anti-foaming agent and α-amylase to aid digestion of non-NDF compounds. Next, 20 ml of NDS and four drops of n-octanol were added to the crucible, which was agitated with pressurised air to mix. Crucibles were heated while another 20 ml of NDS was added. Heating continued until the solution began to boil. Two minutes after boiling was reached a final 10 ml of NDS was added and boiling continued. Two minutes later 2 ml of α-amylase was added and refluxing continued for 55 mins. Crucibles were then taken off the boil and drained by vacuum. Then, 15 ml of deionised water and 2ml of α-amylase was then added, and the crucible agitated for a further minute. Crucibles were then repeatedly rinsed with deionised water and drained by vacuum.

Crucibles were then placed back onto the cold extraction unit and soaked in approximately 30 ml of acetone for 5 mins (without agitation). The acetone was then vacuum extracted through the sinter. This step was repeated three times. Crucibles and samples were then dried at 105˚C to a constant weight, and NDF determined as the remaining material in the crucible, as a proportion of the original sample weight.

Between uses, crucibles were soaked in hypochlorite, in a sonicator bath for 1 hr, rinsed, run through the dishwasher, and furnaced at 525°C.

2.2.3.4 Acid detergent fibre (ADF) (ADF)

Acid detergent fibre was analysed similarly to NDF and in accordance with EN ISO 13906. Firstly, 1.0 g of sample was added to a sintered crucible (porosity 2) along with 0.5 g of sodium sulphite. Samples underwent acetone washing in the cold extraction unit, as per the NDF protocol, to remove lipids.

During the hot extraction stage 45 ml of acid detergent solution (ADS) (Ankom FAD20C) and four drops of *n*-octanol were added to the crucible, which was agitated with pressurised air to mix. Crucibles were heated while another 45 ml of ADS was added. Heating continued until the solution began to boil. Two minutes later a final 10ml of ADS was added and boiling maintained under reflux for 55 mins after which heating was ceases, allow for gradual cooling. Then, 30ml of 90°C deionised water was then added to the crucibles as samples were mixed using pressurised air. Crucibles were then repeatedly rinsed with deionised water and drained by vacuum. Samples were then acetone soaked, rinsed, dried, and weighed, as per the NDF protocol.

Between uses, crucibles were soaked in hypochlorite, in a sonicator bath for 1hr, rinsed, run through the dishwasher, and furnaced at 525°C for at least 3 hrs. After removal from the furnace, crucibles were left to cool to room temperature in a desiccator, before being weighed.

2.2.3.5 Acid detergent lignin (ADL)

Acid detergent lignin determination was conducted subsequent to ADF quantification, using the same sub-samples that went through the ADF procedure. Within the crucibles, samples were soaked in 72% sulphuric acid for 3 hrs, being stirred every hour. Crucibles were then

vacuum drained to remove the acid and then repeatedly rinsed with warm deionised water. Crucibles were then oven dried at 105°C and weighed, before being furnaced at 525°C. ADL was determined as the remaining material, after furnacing, as a proportion of original sample weight.

2.2.3.6 Crude protein (CP)

A method comparison was conducted comparing the Kjeldahl and Dumas methods of crude protein quantification (Appendix 7.6). As a result, CP was analysed by Dumas combustion using a flash elemental analyser (Thermo Scientific Flash EA1112). 5 mg of sample was combusted at 960°C, with exothermic tin combustion at 1600°C, using a carrier gas of helium at 140 ml min^{-1}. Gas chromatography separation used a 0.5 m column packed with active carbon at isothermal 50°C.

2.2.3.7 Crude lipids (CL)

CL was quantified by Soxhlet extraction. 1.0 gram of dried and ground sample was placed in a cellulose extraction thimble (Fisherbrand™ 11754043) into the top of which fat-free cotton wool was placed. The thimble was then placed in a glass extraction chamber which had a cool water condenser fitted to the top. A clean 250 ml round bottom boiling flask was oven dried at 105°c, allowed to cool in a desiccator, and then weighed. It was then filled with 200 ml petroleum ether (ACROS Organics™ 326720025) and attached to the bottom of the extraction chamber. The Soxhlet was left to reflux for 24 hrs and then cool before being dried heated at 103°C, allowed to cool in a desiccator, and reweighed. CL was quantified as the difference in the final and initial weight of the boiling flask, expressed as a percentage of the original sample weight.

2.2.3.8 Non-fibre carbohydrates (NFC)

Non-fibre carbohydrates was determined as the remaining matter after the deduction of CP, CL, NDF, and ash and was calculated a deduction calculation (Equation 2.1).

$$NFC = 1000 - CP - CL - NDF - Ash$$

Equation 2.1 - Equation used to determine NFC content of organic material, all units as grams per kilogram of dry matter.

2.2.4 Elemental micronutrient analysis

Elemental micronutrient analysis was conducted using X-ray fluorescence (XRF) spectroscopy (Bruker TRACER 5i pXRF, USA) on fresh samples that had been dried and ground as specified. Elements measured were, in alphabetical order: aluminium (Al), arsenic (As), bromine (Br), calcium (Ca), cobalt (Co), chromium (Cr), copper (Cu), Iron (Fe), potassium (K), magnesium (Mg), manganese (Mn), sodium (Na), nickel (Ni), phosphorus (P), sulphur (S), selenium (Se), and zinc (Zn). The excitation source was a 4W rhodium target X-ray tube (Max 35μA at 50Kv), and the detector was a proprietary 40 mm^2 silicon drift detector (< 140 eV at 250,000cps (counts per second)). Plant calibrations had previously been established using reference material values measured in-house by total acid dissolution ICP-OES analysis (Towett et al., 2015). Approximately 5 g of sample was placed into sample cups which have a 4 μm prolene film at their base; cups were then placed between the excitation source and detector. Results (%) are an average of continual measurements throughout a 30 second measurement period, and an error was calculated as two standard deviations.

2.2.5 Particle size analysis

Fresh dung (not dried and ground) from cattle fed on silage was analysed for differences in physical composition by measuring the proportions of various particles sizes within the dung. Four sieves were assembled on a sieve shaker (Fritsch Vibratory Sieve Shaker, Analysette 3 pro) in ascending order of mesh size (45 μm, 106 μm, 250 μm, and 2000 μm). Fresh dung of an equivalent 2.5 g DM was placed on the top sieve and the water hood affixed. Water was administered, via spray nozzles in the hood, at a rate of 1L min^{-1} and the sieves shaken at a 1.0 amplitude for 15 min. The top sieve was then removed and the procedure repeated for 5 mins, this was repeated a total of three times so that each sieve was on top for an equivalent period. Sieves were then backwashed using ultra pure (Milli-Q™) water into filter papers (Whatman® 4) (clean, pre-dried at 65°C, and weighed). Filter papers were then dried at 65°C until constant weight. DM weight of each particulate fraction was determined as the increase in weight of the filter paper.

Particle size was not continued forward as an analysis of pasture derived dung. This was due to the time-consuming manner of the protocol; it was therefore deemed to be most productive to focus on other analyses.

2.2.6 In situ dung pat mass

The total mass of fresh dung pats, deposited onto grazed pasture, was determined by calculating the volume of dung pats and the density of the dung using novel and original methodology.

In order to quantify volume, the diameter (Ø) of the dung pat was measured – the mean of three length measurements was taken, the first perpendicular to the North-most field boundary (0˚) and the subsequent two at 120˚ and 240˚. Along each length measured, pat depths were also measured at points 1/6th, 3/6th, and 5/6th out from the centre of the pat to the edge. From a top-down view, the dung pat is split into an outer annulus, inner annulus, and a central circle – each of which represents one-third of the diameter of the entire dung pat. The aforementioned depth measurements thus lie within the centre each of these areas, multiplying these areas by the depth gives an estimation of volumes of those areas (Equation 2.2 – 2.5**,** Figure 2.5).

$$Central\ circle\ volume = x\pi\left(\frac{\emptyset}{6}\right)^2$$

Equation 2.2 - Equation to estimate central circle volume of a dung pat.

$$Inner\ annulus\ volume = y\left[\pi\left(\frac{\emptyset}{3}\right)^2 - \pi\left(\frac{\emptyset}{6}\right)^2\right]$$

Equation 2.3 - Equation to estimate inner annulus volume of a dung pat.

$$Outer\ annulus\ volume = z\left[\pi\left(\frac{\emptyset}{2}\right)^2 - \pi\left(\frac{\emptyset}{3}\right)^2\right]$$

Equation 2.4 - Equation to estimate outer annulus volume of a dung pat.

The sum of these three volumes equals the total volume of the entire pat and is simplified (Equation 2.5).

$$Total\ dung\ pat\ volume = \frac{\pi\emptyset^2}{36}[x + 3y + 5z]$$

Equation 2.5 - Simplified equation to estimate the total volume of a dung pat.

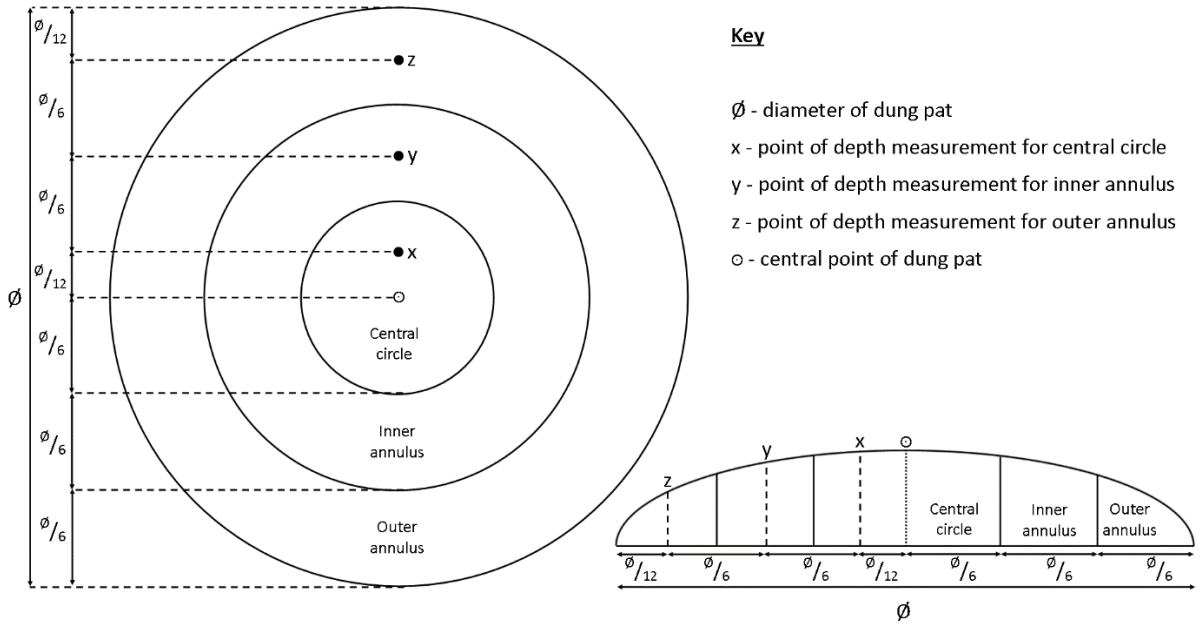


Figure 2.5 - Schematic of a dung pat with points of measurement for volume determination.

Dung density was calculated by utilising the same principles used to quantify soil bulk density. 33 ml sample pots were oven dried at 65°c to a constant weight. Dung from the cow pats was placed into sample pots with great care to avoid compaction; any overfill was carefully removed. The samples were oven dried at 65°c to a constant weight. The weight per litre of dung was then determined as dung density. This density was then multiplied by the volume of the pat to determine the dungs total DM content. These values can then be combined with the aforementioned biochemical and elemental analyses to quantify the total mass of specific dung components.

2.2.7 Statistical analysis

The biochemical components of each farmlet's fresh herbage, silage, and dung were compared using one-way ANOVA with post hoc Tukey tests. The independent variables in each test were the farmlet and material, and the dependent variables were the quantified components.

A general linear model was applied to determine to what extend the two independent variables, dung location (B, G, R) and dung type (b g r) drove dung degradation.

Quadratic polynomial curves were fitted to dung degradation curves for each measured biochemical component. Curves for each group were then compared using an extra sum-of-squares *F*- test to identify significant differences in concentration changes over time. To analyse if the row or column in which dung pats were placed created an experimental bias or edge bias, a two-way ANOVA was completed. Independent variables were: row and column, the dependent variable was total OM (g), and day of sample collection was a covariate.

Micronutrients were compared in reference to minimum requirements and maximum tolerances of beef cattle as outlined by National Research Council (2000).

A one-way ANOVA was conducted to identify is the distribution of particle sizes in dung differed between the different dung types analysed. A post-hoc Tukey test was then used to identify the groupings of any differences.

Dung pat total dry masses from each farmlet, as determined by volume and mass, were compared using a one-way ANOVA. The independent variable was the farmlet, and the dependent variable was dung mass.

2.3 Results

2.3.1 Material composition

When compared, the biochemical components of dung and feeds varied significantly between the different farmlets and materials, supporting hypotheses 1 and 2. It is important to note that ADL is a component of ADF, and ADF is a component of ADL. Therefore the results for these components are intrinsically linked.

Significant differences were found in the OM content of the different feed and dungs across the three farmlets (Figure 2.6). Whilst there is relatively little variation between different sample groups, differences were found due to the small variation and standard error between repeats. With regards to forage, the blue farmlet was had the highest silage OM and was not statistically different to the green farmlet which had the highest herbage OM. For both silage and herbage the red farmlet had the lowest OM. Greater differences were observed between the organic matter of different dung types. When on a silage diet, all dungs had significantly different OM content. Dung produced from a herbage diet was, in all instances, of a lower OM content than dung produces on a silage diet.

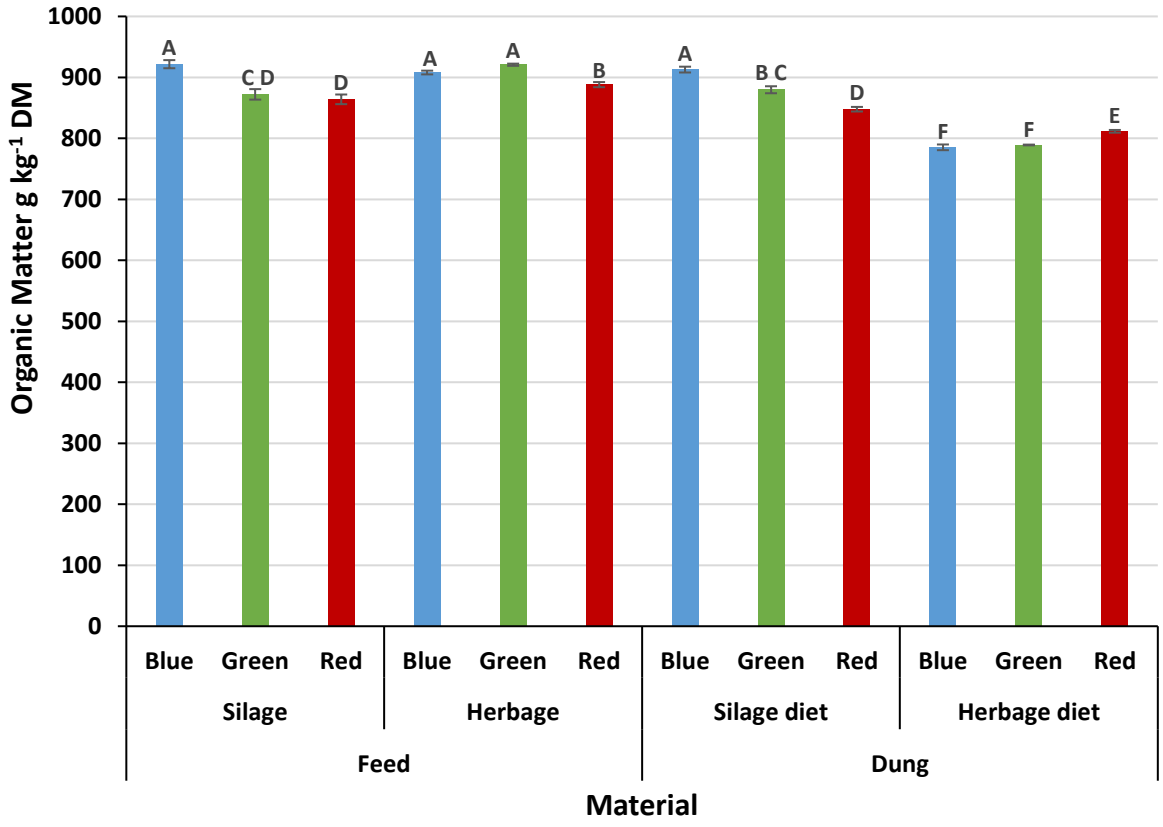


*Figure 2.6 - Organic matter (OM) g kg^{-1} of dry matter, of forage and dung types from three typical UK grazing systems. Statistically significant differences, as identified by ANOVA, were found (*F *= 187.24,* p *< 0.0005). Columns not sharing a same letter are statistically different to one another, as identified by a post-hoc Tukey test. Error bars represent standard error.*

NDF results (Figure 2.7) yielded no significant differences, for each feed type, between farmlets. However, differences were found between the materials, most notably between silage and dung derived from grazed herbage. Dung from cattle fed on silage generally had a higher NDF content than dung from cattle grazing on pasture. NDF concentrations were much more variable between dung types than between feed types.

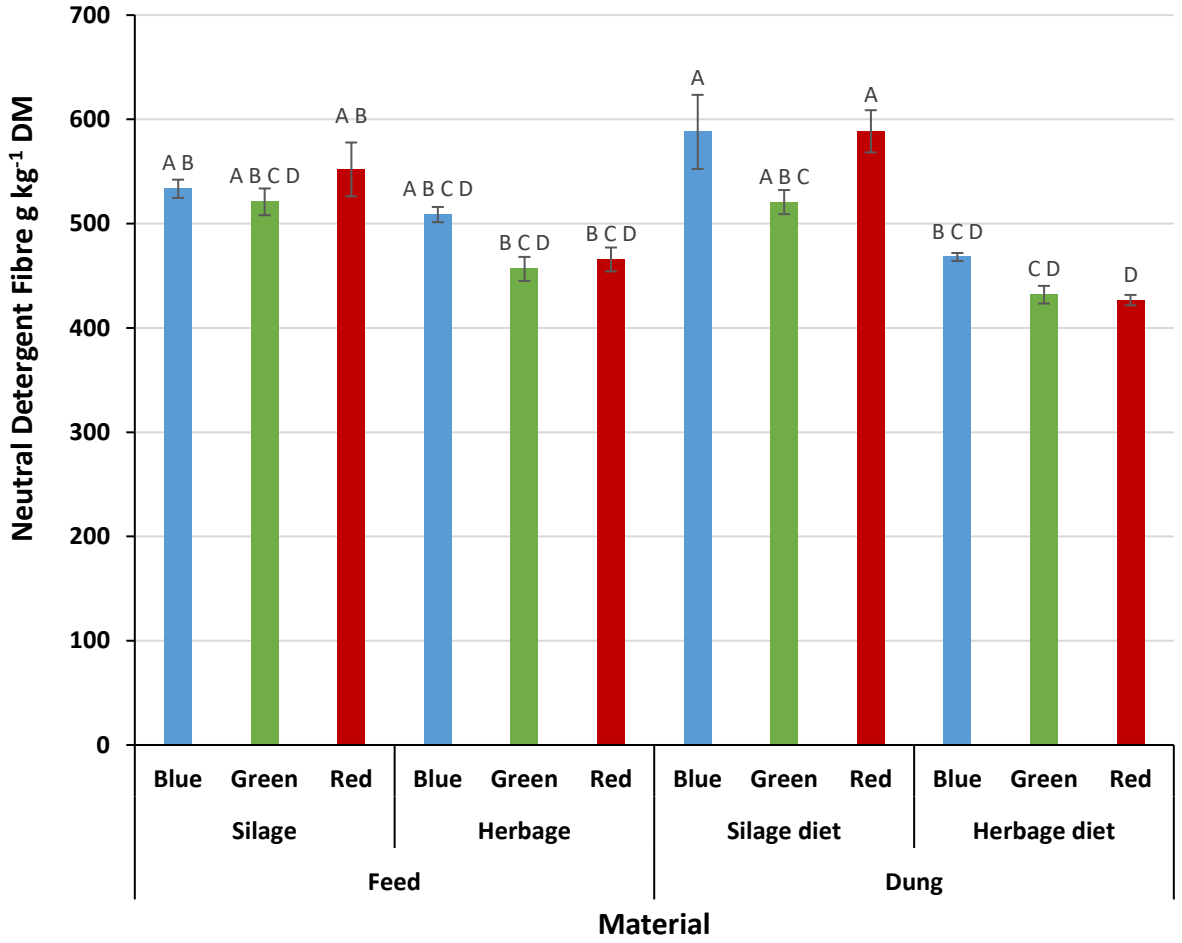


*Figure 2.7 - Neutral detergent fibre (NDF) g kg^{-1} of dry matter, of forage and dung types from three typical UK grazing systems. Statistically significant differences, as identified by ANOVA, were found (*F = 76.02, p < 0.0005*). Columns not sharing a same letter are statistically different to one another, as identified by a post-hoc Tukey test. Error bars represent standard error.*

Acid detergent fibre results (Figure 2.8) showed that ADF was more concentrated in the dung than it was in feed. Half of the feed sample groups were had significantly lower ADF than all the dung samples. Herbage and herbage-derived dung typically had lower concentrations than their silage counterparts. There was also greater variety in feed ADF concentrations, both between repeats within groups and between groups. This variation would reduce the number of statistical differences observed compared to if variation was lower.

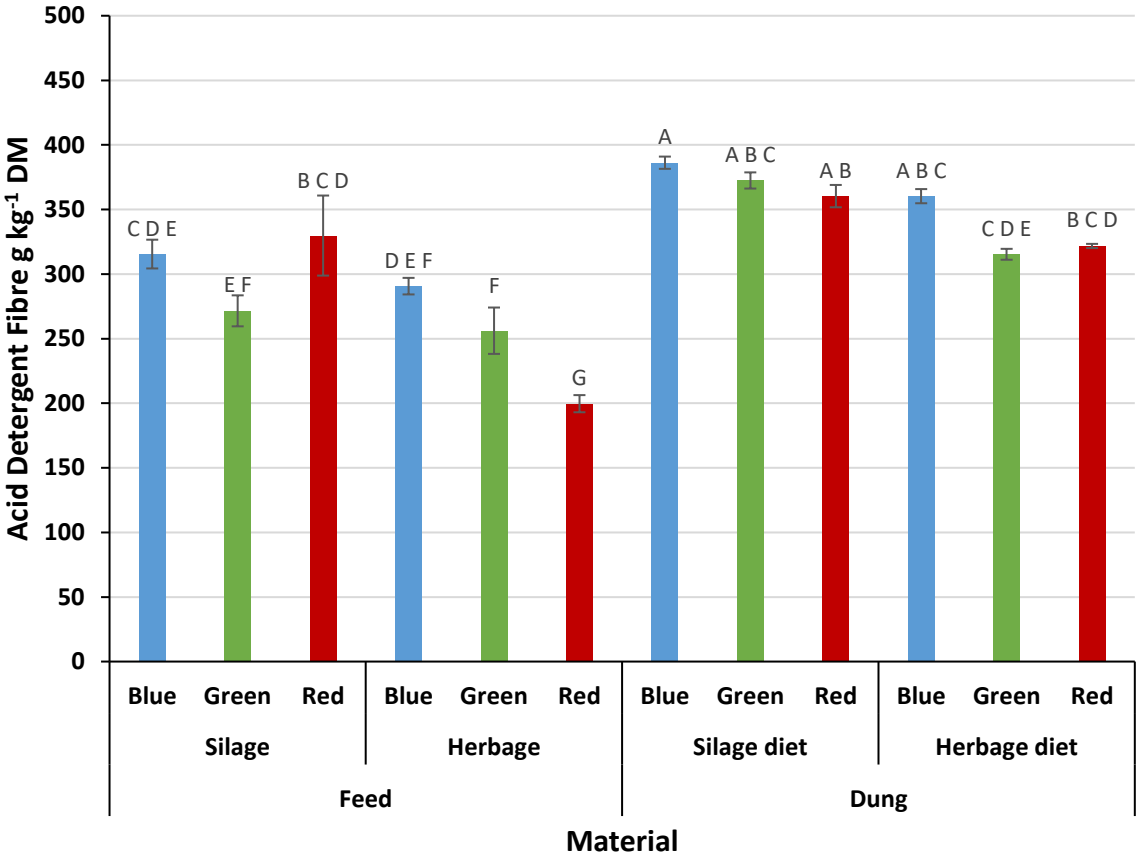


Figure 2.8 - Acid detergent fibre (ADF) g kg^{-1} of dry matter, of forage and dung types from three typical UK grazing systems. Statistically significant differences, as identified by ANOVA, were found (F = 29.47, p < 0.0005). Columns not sharing a same letter are statistically different to one another, as identified by a post-hoc Tukey test. Error bars represent standard error.

Acid detergent lignin concentrations (Figure 2.9) varied significantly between feed and dung. All feed samples had significantly lower ADL concentrations than all dung samples. Between feed samples, herbage appeared to have less ADL than silage, although this was only significant when compared herbage from the red farmlet to the other silages. Dung ADL concentrations followed a clearer pattern for both silage-derived and herbage-derived dung, ADL levels were highest from the blue farmlet and lowest from the red. Overall dung from silage fed cattle had higher ADL concentrations than that produced from herbage fed animals. The standard errors were generally quite low, with the exception of dung from the green farmlet and a herbage diet which showed a high variation.

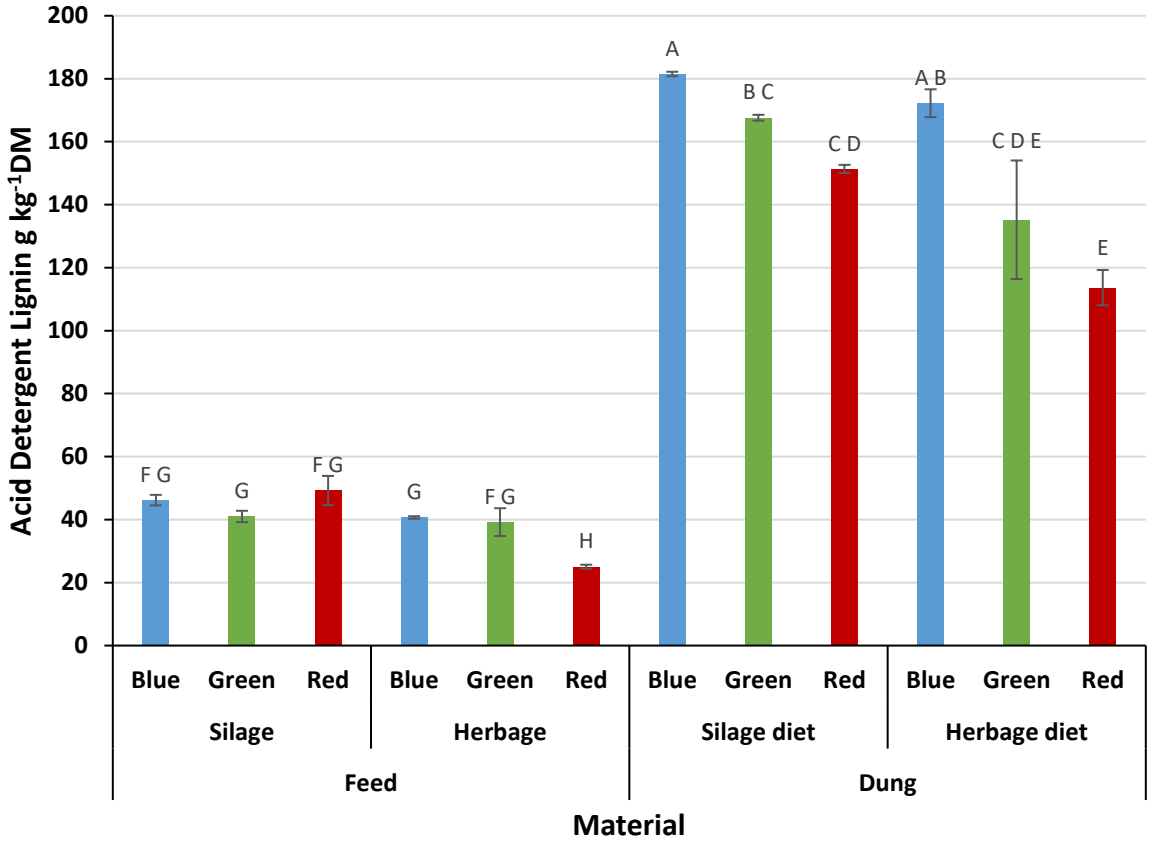


Figure 2.9 - Acid detergent lignin (ADL) g kg^{-1} of dry matter, of forage and dung types from three typical UK grazing systems. Statistically significant differences, as identified by ANOVA, were found (F = 10.12, p < 0.0005). Columns not sharing a same letter are statistically different to one another, as identified by a post-hoc Tukey test. Error bars represent standard error.

Crude protein levels (Figure 2.10) were highly variable between the different materials and also between materials of the same group, derived from different farmlets. Crude protein levels in dung somewhat mirrored those from the equivalent feed, especially when comparing silage and silage-derived dung.

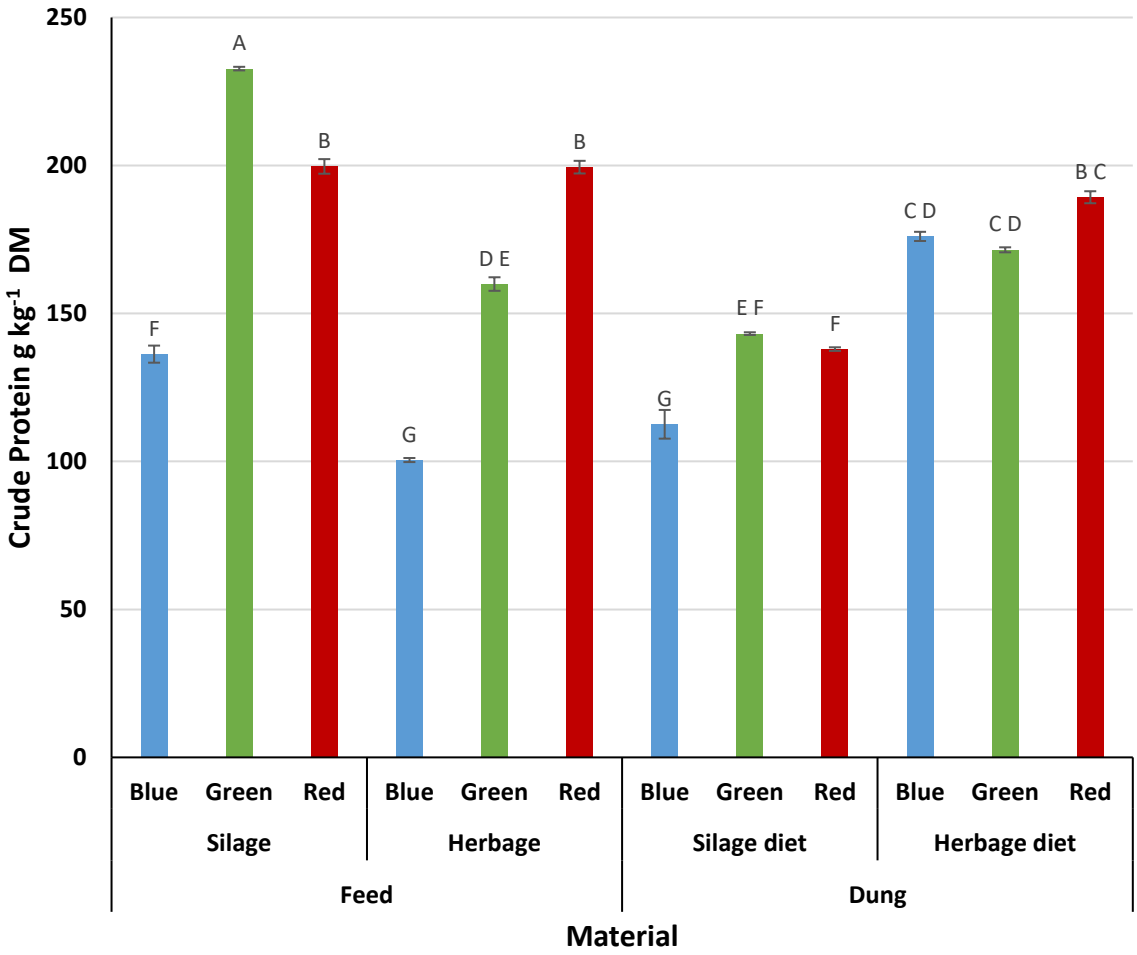


Figure 2.10 - Crude protein (CP) g kg^{-1} of dry matter, of forage and dung types from three typical UK grazing systems. Statistically significant differences, as identified by ANOVA, were found (F = 9.56, p < 0.0005). Columns not sharing a same letter are statistically different to one another, as identified by a post-hoc Tukey test. Error bars represent standard error.

Crude lipids were also highly variable (Figure 2.11), producing relatively high standard errors, especially within herbage samples. Concentrations in dung closely reflected those in the feed from which that dung was generated with higher feed CLi pairing up with higher dung CLi concentrations. In all instances, CLi concentrations were higher in dung than for the dietary equivalent material.

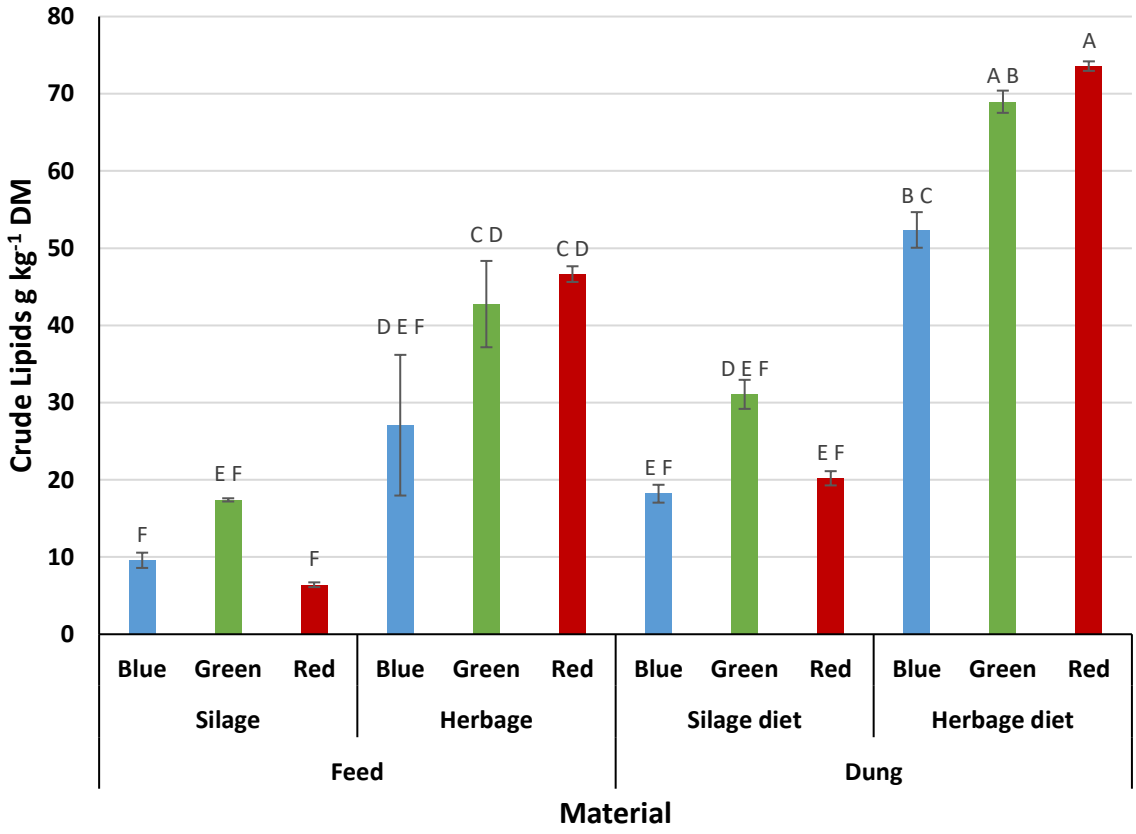


Figure 2.11 - Crude lipids (CL) g kg^{-1} of dry matter, of forage and dung types from three typical UK grazing systems. Statistically significant differences, as identified by ANOVA, were found (F = 1.71, p < 0.0005). Columns not sharing a same letter are statistically different to one another, as identified by a post-hoc Tukey test. Error bars represent standard error.

In all instances, NFC levels (Figure 2.12) were higher in feeds than in dung, however this was not always significant. Dung derived from a herbage diet had the lowest NFC concentrations compared to other materials and was significantly different to all other sample groups apart from dung derived from silage from the red farmlet. Variation in NFC was relatively high compared to other measured components.

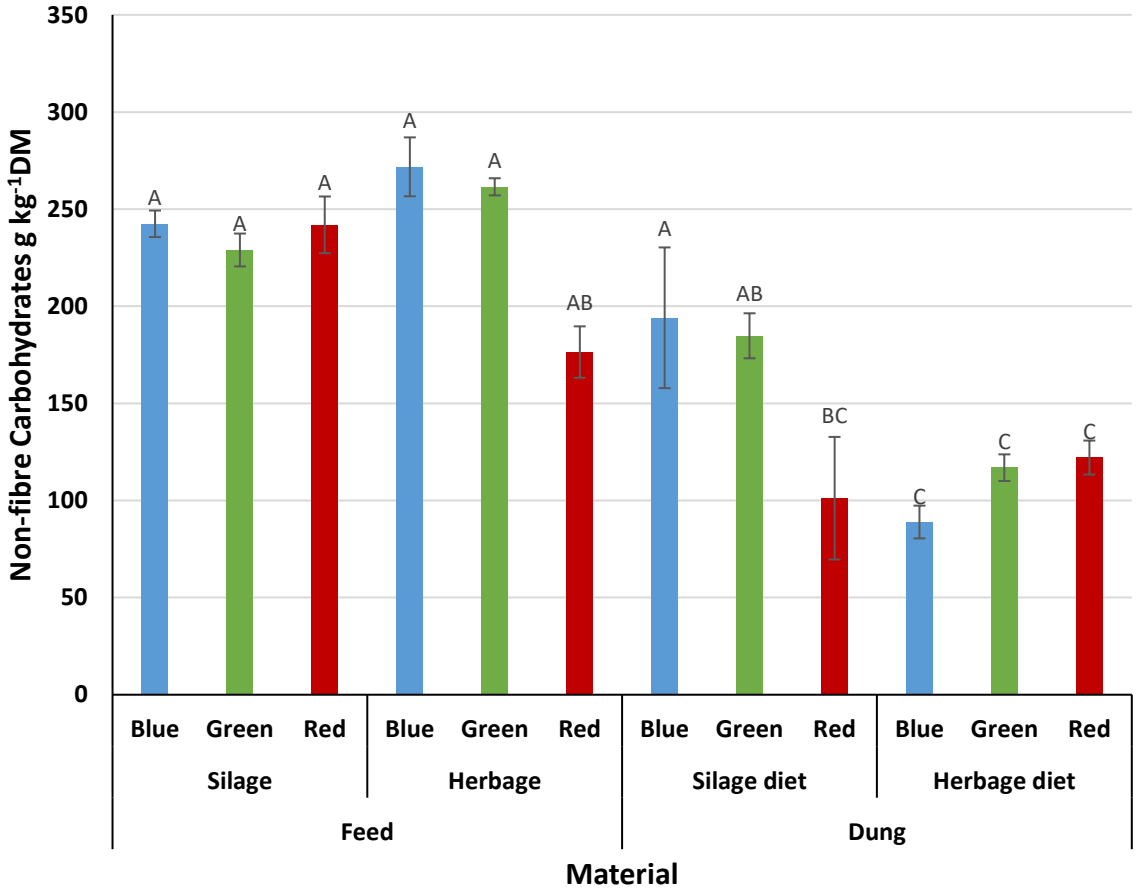


*Figure 2.12 - Non-fibre carbohydrate (NFC) g kg^{-1} of dry matter, of forage and dung types from three typical UK grazing systems. Statistically significant differences, as identified by ANOVA, were found (*F = 26.58, p < 0.0005*). Columns not sharing a same letter are statistically different to one another, as identified by a post-hoc Tukey test. Error bars represent standard error.*

Elemental analysis by XRF successfully quantified 17 of the 19 elements within the capability of the device. Bromine and selenium, in all instances, were below the limit of detection (LOD) of 1ppm (Table 2.2). An associated error table is available in Appendix 7.5.

Table 2.2 Results of XRF analysis showing concentrations of elements (ppm) in silage, herbages, and subsequent dungs, over the three farmlets of Rothamsted Research's North Wyke Farm Platform. For forages, Results are colour coded by their value, in reference to their minimum and maximum concentrations as per National Research Council (2000). <LOD refers to results below the limit of detection. Whilst for many samples it was possible to determine if <LOD samples were above or below thresholds, it was not possible for all samples. Therefore, <LOD samples were left colourless.

			Concentration of element in material (ppm)											
			Feed						Dung					
			Silage			Herbage			Silage diet			Herbage diet		
	Min	Max	B	G	R	B	G	R	B	G	R	B	G	R
Al	n/a	1000	< LOD	< LOD	1121	1131	< LOD	< LOD	2539	2935	1946	753	870	2831
As	n/a	50	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	1
Br	n/a	200	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
Ca	5500	n/a	26417	30449	30314	16458	26552	39471	11637	9475	11486	9498	13615	16399
Co	0.1	10	1	< LOD	< LOD	< LOD	1	2	15	4	3	4	2	4
Cr	n/a	1000	25	< LOD	< LOD	< LOD	< LOD	< LOD	49	38	36	44	17	69
Cu	10	100	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	284	13	< LOD	28	24	< LOD
Fe	50	1000	441	444	2066	1036	593	301	3444	3523	3622	1624	1221	4111
K	6000	30000	4455	5629	4231	5800	5811	6982	12303	17209	10853	21993	12781	10279
Mg	1000	4000	2137	3132	2484	1359	3192	3327	6199	5446	4735	3093	4154	2815
Mn	20	1000	295	256	442	259	309	249	697	529	840	948	582	777
Na	600	n/a	16259	27587	< LOD	< LOD	< LOD	34534	19034	< LOD	< LOD	< LOD	18649	< LOD
Ni	n/a	50	2	2	< LOD	< LOD	< LOD	4	< LOD	< LOD	< LOD	< LOD	1	< LOD
P	2200	100000	2200	2875	2598	2514	3053	3482	6057	7243	7139	5248	8369	5324
S	1500	4000	1485	2231	1910	2235	2432	3265	2723	2388	2533	1914	2602	2116
Se	0.1	2	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
Zn	30	500	20	32	< LOD	14	< LOD	30	91	85	39	73	160	74

2.3.2 Dung degradation

A two-way ANOVA showed no statistically significant difference in total OM content between different rows ($F = 0.31$, $p = 0.905$) and columns ($F = 0.56$, $p = 0.733$) of field plots. Quadratic polynomial curves for total organic matter were significantly different between the nine groups, as determined by an extra sum-of-squares F-test ($F = 6.140$, $p < 0.0005$) (Figure 2.13; Appendix 7.4). Clear grouping and patterns can be seen across both independent variables. Dung placed on the green farmlet degraded the slowest, followed by red, and then blue. Within each of those groups, the order of the rate of degradation of each dung type was the same, dung from the blue system degrading slowest, then red, then green. A comparison of decay rates, by general linear model, attributed 69.4% of degradation to be driven by field site (B, G, R) and 4.4% to be driven by dung type (b, g, r) ($p < 0.0005$). Results support the acceptance of Hypothesis 3.

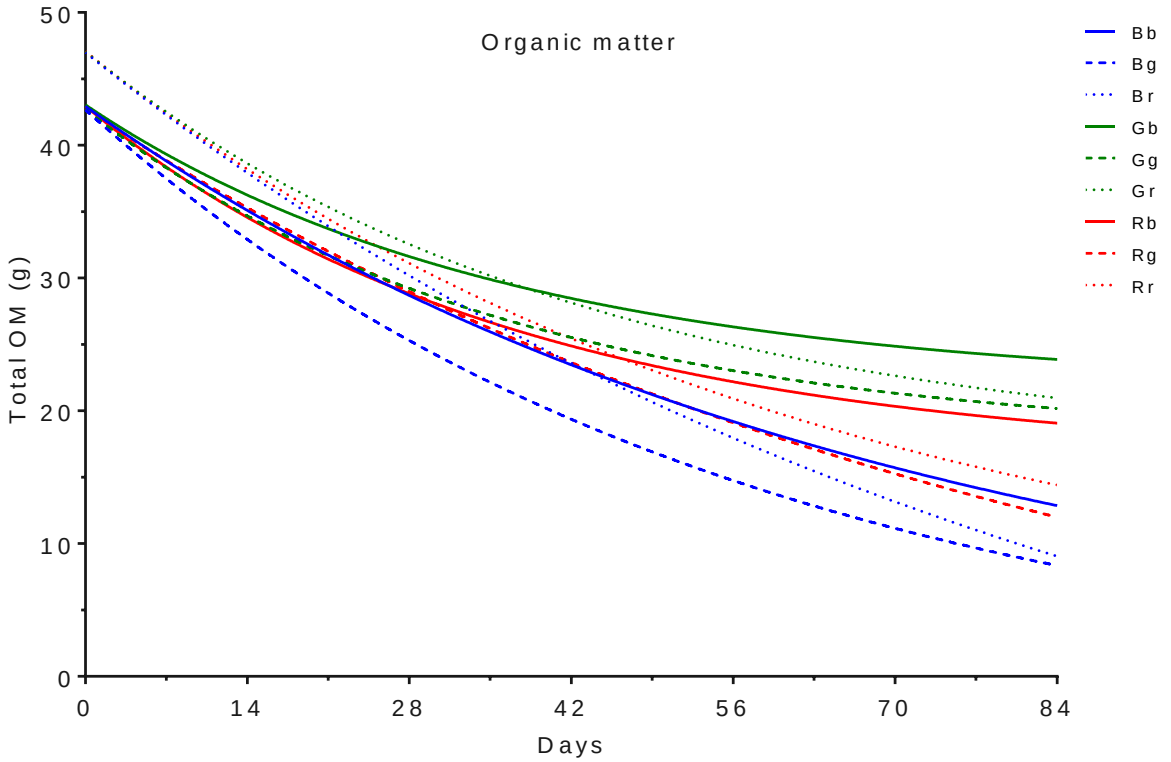


Figure 2.13 - Quadratic polynomial curves of organic matter concentration (% of DM), over 84 days, for three field plots (B, G, R) with three distinct dung types on each (b, g, r) forming 9 groups (Bb, Bg, Br, Gb, Gg, Gr, Rb, Rg, Rr). For raw data, see Appendix 7.4.

Neutral detergent fibre concentrations increased over time for all groups. Concentrations increased most slowly in dung located on the green farmlet, from approximately 43% to 54% and most rapidly in the red farmlet, reaching more than 75% for samples Rb and Rr. Some grouping is apparent with the NDF concentrations lowest in dung placed on the green farmlet by the end of the 84 day experiment. Curves for each data set were not significantly different (F = 1.291, p = 0.1882) (Figure 2.14; Appendix 7.4).

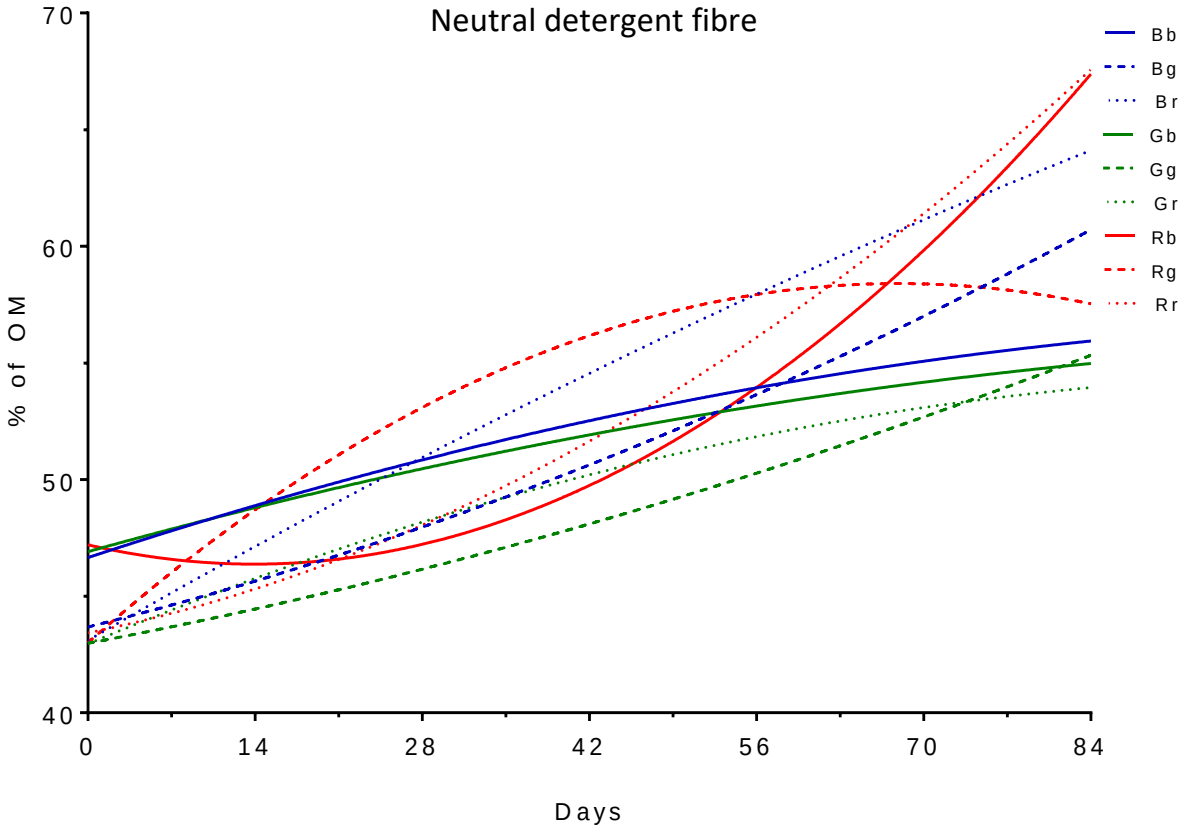


Figure 2.14 - Quadratic polynomial curves of neutral detergent fibre concentration (% of DM), over 84 days, for three field plots (B, G, R) with three distinct dung types on each (b, g, r) forming 9 groups (Bb, Bg, Br, Gb, Gg, Gr, Rb, Rg, Rr). For raw data, see Appendix 7.4.

Acid detergent fibre concentrations increased continuously with clear grouping, by location, becoming apparent over time (Figure 2.15; Appendix 7.4). Whilst dung degrading on the red farmlet had ADF concentrations of approximately 70% after 84 days, concentrations from dung on the green farmlet were approximately 40% after the same period. By the end of the experimental period at each location the red dung had the highest ADF concentration followed by green and then blue with the lowest. Curves for each data set were significantly different ($F = 3.846$, $p < 0.0001$).

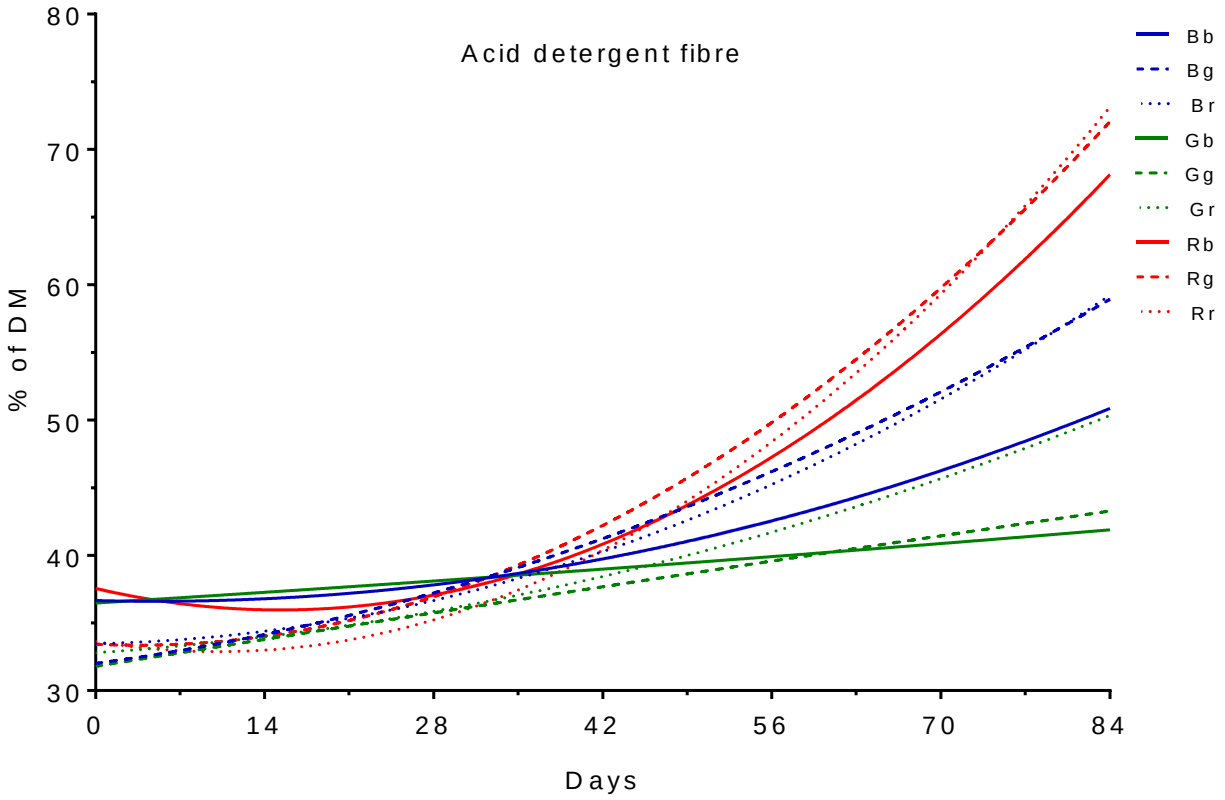


Figure 2.15 - Quadratic polynomial curves of acid detergent fibre concentration (% of DM), over 84 days, for three field plots (B, G, R) with three distinct dung types on each (b, g, r) forming 9 groups (Bb, Bg, Br, Gb, Gg, Gr, Rb, Rg, Rr). For raw data, see Appendix 7.4.

Acid detergent lignin concentrations initially increased towards 28 days before a steady decline for the remainder of the experiment (Figure 2.16; Appendix 7.4). Some grouping was present by dung type, although not as clearly as with components, with concentrations in dung derived from the red farmlet increasing rapidly over the first half of the experiment, followed by an equally rapid decline. Curves for each data set were significantly different (F = 3.069, $p < 0.0001$).

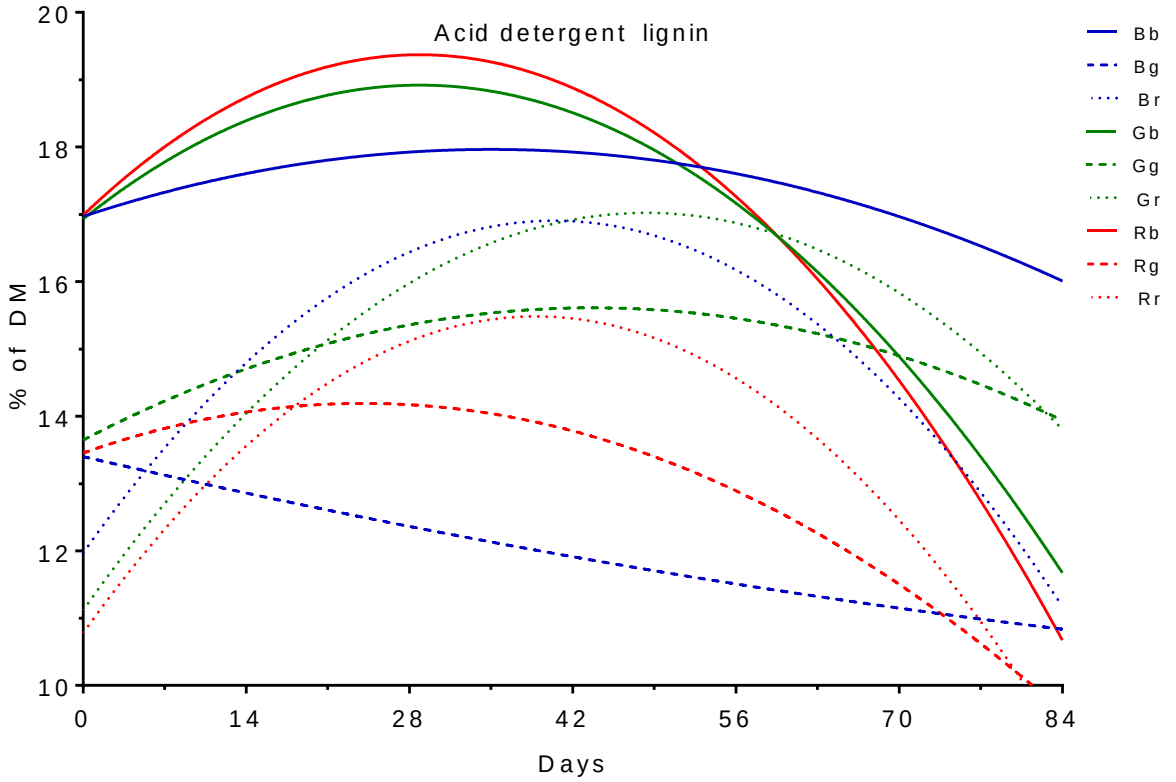


Figure 2.16 - Quadratic polynomial curves of acid detergent lignin concentration (% of DM), over 84 days, for three field plots (B, G, R) with three distinct dung types on each (b, g, r) forming 9 groups (Bb, Bg, Br, Gb, Gg, Gr, Rb, Rg, Rr). For raw data, see Appendix 7.4.

As a percentage of total DM, CP decreased gradually over time, after small initial increase and plateaus (Figure 2.17; Appendix 7.4). Some grouping was present, becoming more apparent over time, with crude protein concentration reducing most rapidly in dung located on the red farmlet, reducing to as little as <8%, compared to a slower reduction on the green farmlet to around 16%. Curves were significantly different for each data set ($F = 4.123$, $p < 0.0001$).

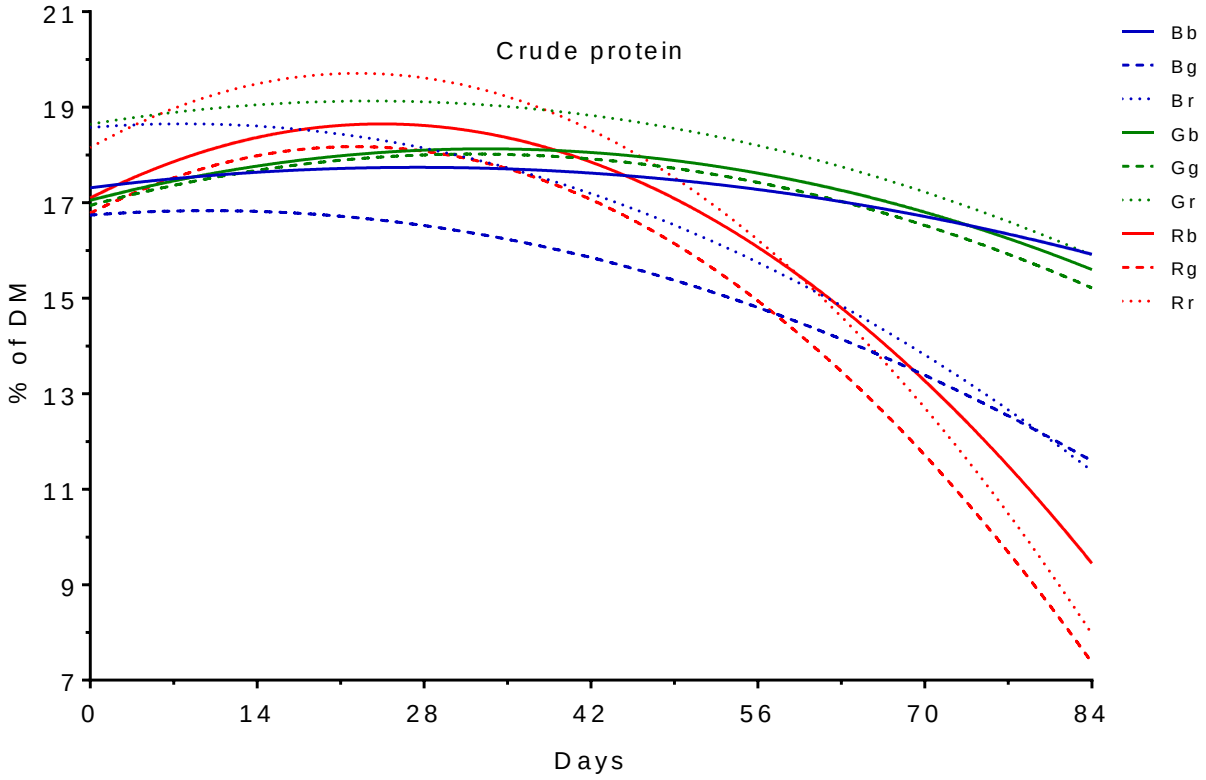


Figure 2.17 - Quadratic polynomial curves of crude protein concentration (% of DM), over 84 days, for three field plots (B, G, R) with three distinct dung types on each (b, g, r) forming 9 groups (Bb, Bg, Br, Gb, Gg, Gr, Rb, Rg, Rr). For raw data, see Appendix 7.4.

Crude lipids levels decreased over time, rapidly at first with the rate decreasing and almost plateauing towards 84 days (Figure 2.18). Rates of decrease were greatest for dung located on the red farmlet and from dung derived from animals on the blue farmlet. The initial CLi concentrations of dung appeared to have only a relatively minor influence to the concentrations observed at the end of the experimental period. Curves were significantly different for each data set ($F = 2.558$, $p = 0.0006$).

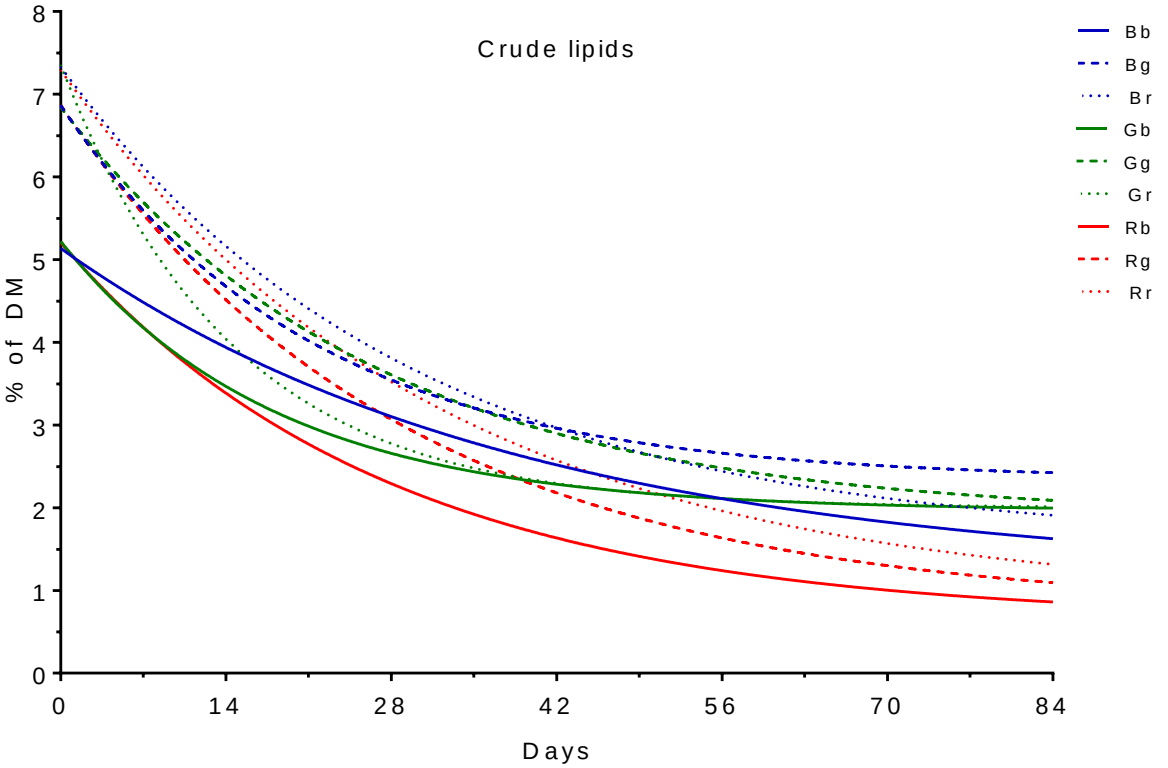


Figure 2.18 - Quadratic polynomial curves of crude lipid concentration (% of DM), over 84 days, for three field plots (B, G, R) with three distinct dung types on each (b, g, r) forming 9 groups (Bb, Bg, Br, Gb, Gg, Gr, Rb, Rg, Rr). For raw data, see Appendix 7.4.

Non-fibre carbohydrate concentrations consistently decreased and in many cases, fell to undetectable levels (Figure 2.19; Appendix 7.4). This rate of decrease was least in dung located on the green farmlet. Curves for each data set were not significantly different (F = 1.008, p = 0.4626).

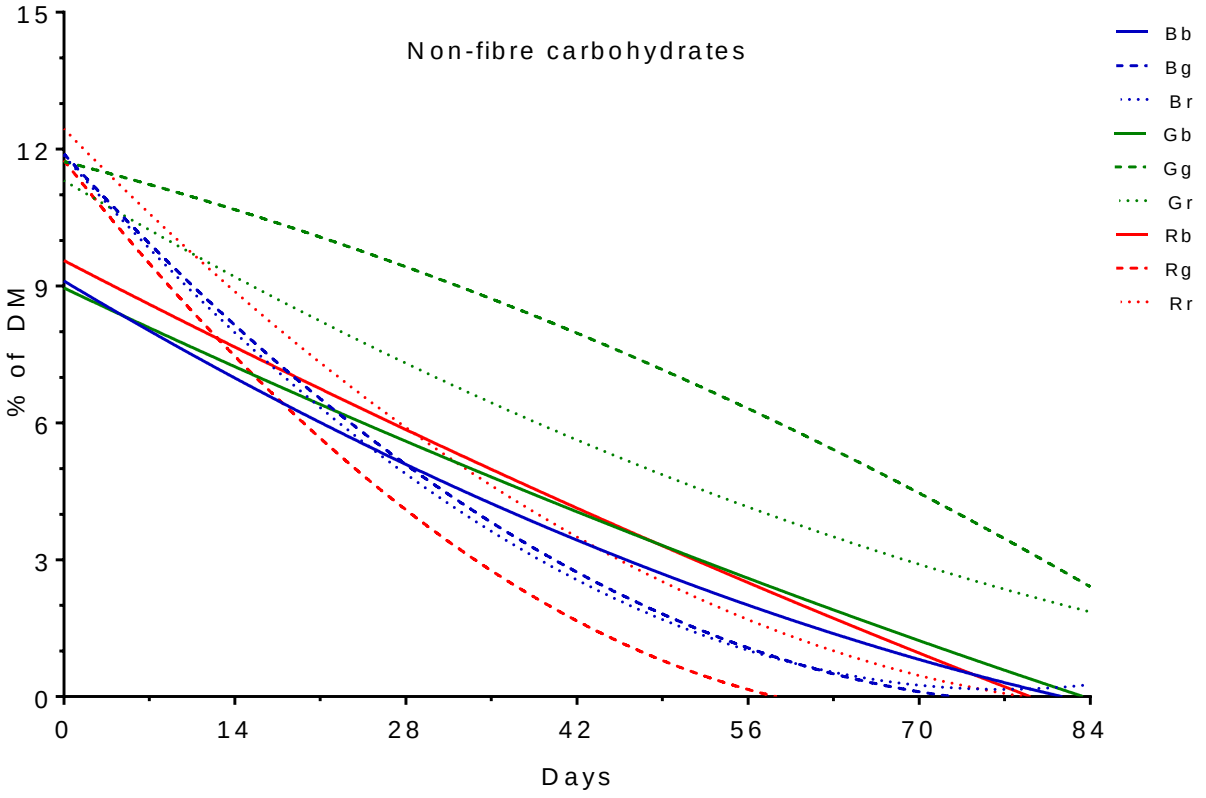


Figure 2.19 - Quadratic polynomial curves of non-fibre carbohydrate concentration (% of DM), over 84 days, for three field plots (B, G, R) with three distinct dung types on each (b, g, r) forming 9 groups (Bb, Bg, Br, Gb, Gg, Gr, Rb, Rg, Rr). For raw data, see Appendix 7.4.

2.3.3 Particle size

One-way ANOVA and 2-sample *t*-tests found statistically significant differences in the particle size composition of the different dungs examined (Figure 2.20). For all samples, soluble material was the largest particle component of the dung and material >2000 µm the smallest component. Significant differences were found between at least one pairing in every particle size apart from 45-106µm.

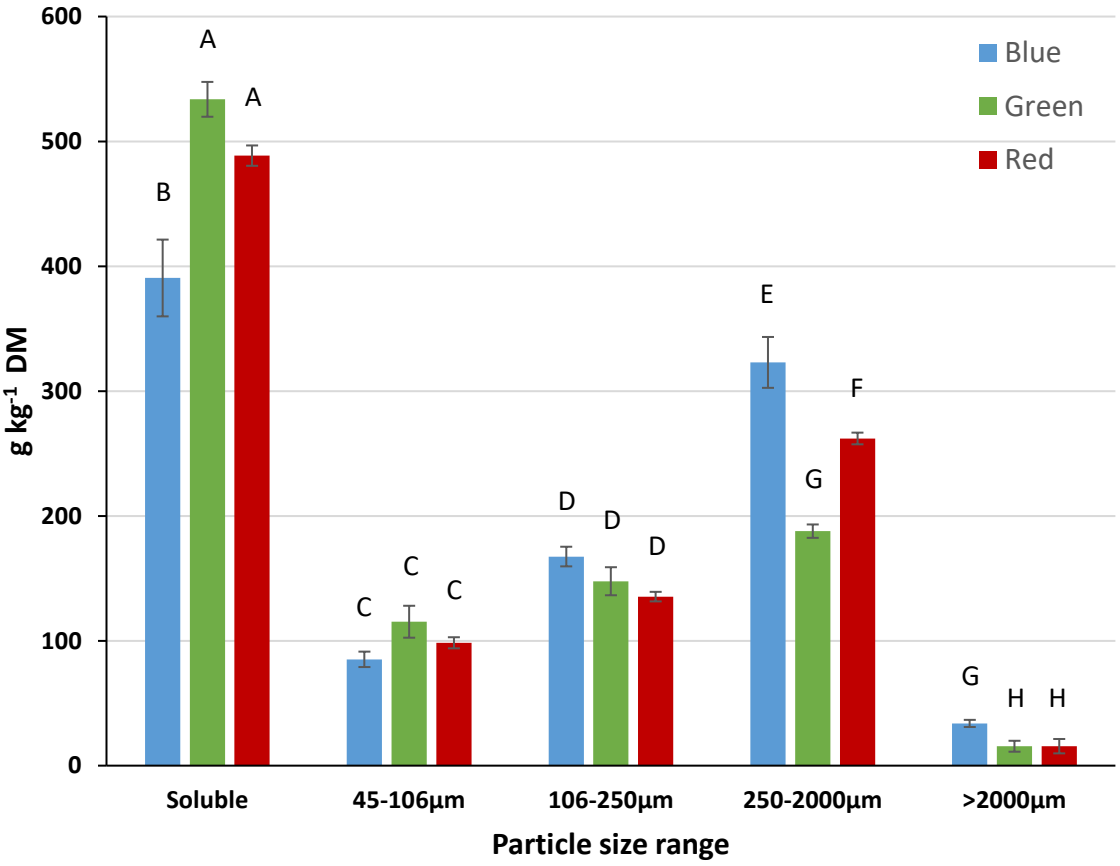


Figure 2.20 - Dung derived from cattle fed on three distinct silage types, each derived from a unique farmlet, (Blue, Green, Red), were compared for differences in particle size composition. Particle size compositions quantified were: soluble, 45-106µm, 106-250µm, 250-2000µm, and >2000µm. A One-way ANOVA found statistical differences between dung types for three of the four particle sizes (soluble, 250-2000µm, and >2000µm – F = 13.31, p = 0.002, F = 3.09, p = 0.095, F = 3.88, p = 0.061, F = 29.52, p < 0.0005, F = 5.51, p = 0.027 respectively). Columns not sharing the same letters are significantly different to each other, as identified by a posthoc Tukey test.

2.3.4 Dung pat mass

Differences in total DM of fresh dung pats, as determined by One-way ANOVA, were not significant ($F = 0.70$, $p = 0.507$) (Figure 2.21). However, there is a greater than 20% difference in mean dung pat mass between the red and green farmlets, with a much greater range in pat size observed on the green farmlet than on the red. Results reject the hypothesis 4 in favour of the null hypothesis.

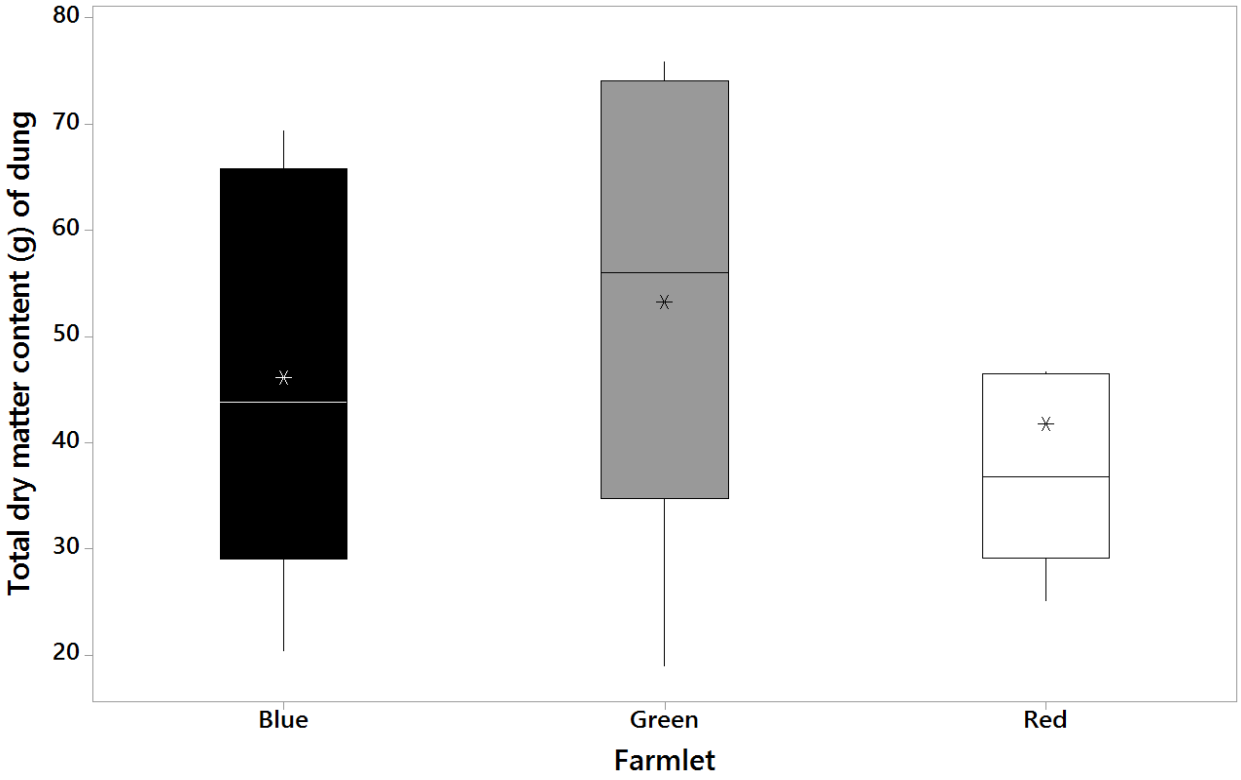


Figure 2.21 - Boxplots of total dry matter (DM) (g) of fresh dung pats, naturally deposited onto pasture, derived from each of the three farmlets. Mean values, left to right, are 46.1g, 53.3g, and 41.8g. Asterisks represent mean.

2.4 Discussion

Pasture composition of the three different systems produced significant differences in the chemical and physical composition of cattle dung. While these differences did manifest themselves as differences in the rate of dung degradation, the pasture on which a dung pat was located was found to be a greater driver of degradation than the biochemical composition of the dung. Evidence is found supporting the hypothesis that pasture

management, with regards to pasture composition, can have a significant impact on factors central to the nutrient cycling process. Such differences when scaled up to the amount of dung produced per animal per year, may produce a significant change in the nutrient dynamic cycling within the system.

2.4.1 Nutrition analysis

The significant differences found in feed composition infer the possibility of consequential variations in the nutritional and health status of animals within the different farmlets. These differences may not necessarily manifest themselves as significant changes in liveweight or other health measures, possibly because the diets of each system are generally balanced, or because some deficiencies and overabundances only manifest in certain circumstances. For example, selenium deficiency can increase disease susceptibility, but only in situations where there is a significant disease challenge to the animal (Boyne and Arthur, 1981; Koller et al., 1983; Stabel et al., 1989). In addition, micronutrient inadequacies can be caused and exacerbated by stress (Stabel et al., 1989), cattle reared on the North Wyke Farm Platform are reared under 'best practice' management, meaning animal stress and its consequences are minimised as much as possible.

2.4.1.1 Macronutrients

Acid detergent fibre concentrations in dung were higher than in feed. ADF is relatively indigestible, particularly due to its lignin component which is not broken down by the animal due to the cross-linking and lack of liable bonds. This is reflected by the observed higher ADL levels in dung than feed. Nevertheless, ADF is a valuable component of feeds by facilitating the healthy transition and passage of nutrients through the rumen. Conversely, NFC levels were lower in dung than in feed, NFC represents highly digestible carbohydrates and sugars, which are readily utilised by the animal (Hoover and Stokes, 1991).

Whilst it is not possible to conclude that one particular forage is of better quality than any other within the study, the breadth of significant differences observed is important. The potential impact of these differences could be far-reaching and drive important biological mechanisms relating to animal health and performance.

Whilst herds from each farmlet did not significantly different in their cold carcass weights (Appendix 8.7) that does not mean that nutritional differences have no impact. Nutritional

differences may be masking greater differences in performance than are observed, they may drive unmeasured factors relating to meat quality, or they may only become apparent in times of stress (i.e. a disease outbreak).

2.4.2 Micronutrients

A number of key micronutrient imbalances were observed in forages when compared to recommended thresholds (National Research Council, 2000). Two forage samples were recorded to have an overabundance of iron, whilst for blue farmlet herbage this was only 4% in excess of the top threshold, red farmlet silage was 107% over. At high levels, iron can be toxic to cattle, reducing feed intake and causing diarrhoea and even hypothermia (Dillman et al., 1980; National Research Council, 2000). Given that silage is the predominant feed during winter, the potential for hypothermia and reduced body temperature mediation is of particular importance. Of the six forages, five showed potassium concentrations below the minimum threshold. Whilst severe potassium defficiencies are not common in cattle; they do have the ability to reduce feed intake and weight gain (Devlin et al., 1969). The high absorption of dietary potassium, through the rumen, means that deficiencies can occur quickly. Forage zinc concentrations were below the lower threshold for two of the forage samples (blue farmlet herbage and silage), below limits of detection for two others (red farmlet silage and green farmlet herbage), and the two samples within range (green farmlet silage and red farmlet herbage) were only so by small amounts.

In the majority of cases, results below the limit of detection were likely to also be below the minimum threshold set for that particular element. However, as detection limits were not known it was not always possible to confirm that with certainty. For example, two positive zinc concentrations were below the minimum threshold. Therefore it can be deduced that all <LOD samples are below those values and therefore also below the minimum threshold. Conversely, sodium samples below <LOD can only certainly be said to be below the lowest positive value of 16259 ppm, which is far greater than the minimum threshold of 600 ppm. Testing of a range of concentrations of reference materials, for each element, would provide information of the detection limits of the XRF apparatus, therefore allowing for more information to be derived from results that fall below these limits.

2.4.3 Dung degradation

Results show that dung pat location was a greater driver of degradation than dung composition. This means that parameters that vary between the field sites are driving the degradation, such as invertebrate activity and growth of flora. In the blue farmlet, in which dung degraded at the greatest rate, it was observed that legumes were pushing through the dung pats as they grew, concurrent with observations by Weeda (1967), whilst in the other field plots, this was rarely the case. Fungi were also notably more visible in these pats than in those on the other two farmlets, which may have important implications for GIN survival due to the nematophagous nature of many fungi (Larsen et al., 1994; Larsen, 2000; Waller and Larsen, 1993). Over time the biochemical composition of dung pats significantly change. Immediate trends emerged with CL and NFC concentrations quickly diminishing and, after an initial lag phase, protein. These compounds share two characteristics; they are all high in energy and all scarce within the dung. The culmination of this is that dung pats became more fibrous in nature as these other components diminished. Fibrous components of dung may form large and complex structures which provide strength against abiotic degradation. Conversely, other components, such as protein and lipids are more predominant in the liquid phase of dung, which is highly susceptible to abiotic events such as rainfall. This is also the phase that invertebrates typical feed on, whilst fibre is of comparatively low nutrition to invertebrates. The culmination of these effects is the persistent of fibrous dung components over time and the relatively more rapid removal of non-fibre components of dung.

Whilst significant differences were found, between groups, in the temporal concentrations of many of the dung components, the general trends of groups were consistently similar. This strengthens the external validity of findings, suggesting that these trends are likely to be seen elsewhere with other dung types and environments, however, the extent of these trends may differ more greatly than in this example. The significant differences in degradation rates have implications for nutrient cycling. Taking the two most extreme examples in the study, over 84 days Gb lost 45% of OM whilst Bg lost 80%. The consequence for this is that nutritional components of Bg are more quickly available for organisms within the system, in a more consistent manner, this harbours the potential to enhance soil OM, the soil microbiome, and consequently pasture productivity. This may also have an impact on the movement of pathogens within the system. Invertebrates and fungi have been found to consume and bury

pathogens of livestock, including GINs, reducing the risk of infection (Duffield and Young, 1985; Fincher, 1973, 1975; Larsen et al., 1994, 2000; Phillips et al., 2003; Waller and Larsen, 1993). Such organisms are also positively associated with dung degradation (Barth et al., 1994b, 1995; Floate, 1998b; Lussenhop et al., 1980) and, through similar underlying mechanisms, the degradation of cattle dung may be an indicator or driver of reduced risk of pathogenic infection in livestock.

The role of invertebrates in dung degradation is particularly interesting in relation to the use of veterinary medicines. Invertebrates play a significant role in the degradation of dung. However, this can be impeded by the use of veterinary medicines, such as anthelmintics, which can possess insecticidal properties (Adler et al., 2016; Barth et al., 1993, 1994a; Floate, 1998a; Lumaret et al., 2012; Wall and Beynon, 2012; Wall and Strong, 1987). In addition to pasture management, another key aspect of farm management is the chosen level of veterinary intervention, particularly for parasitic diseases. It is common for farms to whole-herd dose on a regular basis, while at the other end of the spectrum, organic farms only treat when there is a significant risk to animal health, with targeted selective treatment as a more intermediate approach. Such variations in the management of veterinary medicines, therefore, impact dung degradation. Combined with the observed differences in dung degradation, brought about by pasture type, this could create significant variability, between diverse farming systems, to the rate that dung degrades and nutrients cycle within their specific system. The impact that the management of veterinary medicines has upon dung fauna has been well-studied at causal level (Barth et al., 1994a; Beynon, 2012; Floate, 1998a; Madsen et al., 1990a; Römbke et al., 2009; Wall and Beynon, 2012; Wall and Strong, 1987), and there is, therefore, the necessity for a more detailed approach, especially in relation to the nuanced differences in treatments.

While no statistically significant differences were found between dung pat masses across the three systems, it is notable that the mean and distribution for dung pats on the red system were much smaller than that of the green system. Given that difference and the p-value of 0.246, there is the potential that there could be a real-world difference. A larger and more longitudinal sample would be necessary to show this conclusively. Such an experiment could further be enhanced by the incorporation of additional and more varied diets into the experimental design, which could exacerbate differences. This would be well complemented

by observational research into the defecation rates of different cattle, providing more resolution as to the exact dynamics of natural dung deposition onto pasture.

2.4.4 Further work

Despite the differences between the pastures, there were still similarities in the diets of each herd and therefore the biochemical and physical properties of the dung. A broader range of dung types, such as those derived from grain-based diets, or from animals suffering from diarrhoea, would allow further work to more comprehensively assess the impact of pasture type upon dung dynamics. In such an instance, as more varied diets were included more diversity in dung biochemistry would be observed, which may further manifest itself in physical consistency, which is a reported driver of dung degradation (Weeda, 1967). For example, dung with lower viscosity will have a greater surface area-to-volume ratio, absorb into soil more easily, and will be more sensitive to abiotic degradation. A consequence of this could be increased nutrient losses in runoff (Eghball et al., 1997). In other and potentially extreme examples, dung composition may become a more significant driver. For example, dung with very little structure, such as that caused by diarrhoea (a common clinical sign of parasitemia), is likely to degrade quickly due to increased mobility and absorbency.

When attempting to understand livestock production systems as a whole, it is essential to monitor feed and dung composition. This is particularly pertinent to system nutrient cycling and animal health, both of which are core considerations and metrics within pasture-based livestock systems. Dung composition is a vital aspect of system nutrient dynamics through the deposition and turnover of significant quantities of biochemicals. Increased understanding of these dynamics would allow for assessment of nutrient use and waste and the environmental impact of the system on the surrounding environment, such as the impact on water sources. The result of this is increased efficiency both economically and environmentally, by minimising energy losses and sub-optimal feed conversion. This must be balanced with animal health considerations, which have to be central to decision-making. However, the three systems examined yielded no significant difference in cattle carcass sale prices. This provides reassurance that differences in pasture type can be made without impacting animal performance – so long as appropriate caution is taken.

2.4.5 Analysis of agricultural materials

The value of the work is two-fold, with benefits towards agricultural science in general, but also by the novel development and application of methods for agricultural materials.

Forage analysis is a well-studied and established field, and the methods have been refined and scrutinised over the years. However, the overwhelming focus has been on the analysis of feed (hence the name, 'forage analysis'), yet there is scope and use for these methods to be used to analyse other materials. Quantification of dung components goes towards providing a more complete picture of animal nutrition, by providing information regarding which nutrients, and to what extent, are most utilised and most passed by the animal. Furthermore, these results provided valuable information regarding the physical composition of dung and how that changes over time with the turnover of nutrients into the environment.

Although not taken forward, the methodology used for dung particle size analysis is relatively novel, based on pre-existing principles used elsewhere in agricultural and environmental science. Particle size differences have the potential to impact the physical nutrient flows within agricultural systems by influencing the transport of nutrients in water, for example. Particle size analysis has been utilised in the study of slurries. However, these principles have not been readily applied to dung. While dung is typically more solid than slurry; its physical properties are subject to change with the absorption of moisture during rainfall.

The estimation of dung pat mass was a technical success. While there is no comparable protocol available to validate this method, the underlying mathematical principles are well-founded. The method is also simple, requiring few resources. This method could be more widely applied to estimate the input of dung, by cattle, on pasture, providing supporting information for the assessment of total system nutrient inputs. Given the comparative lack of detailed focus that is placed upon naturally deposited dung as a fertiliser, compared to manually applied fertilisers, it seems that there is significant room for advancement in our understanding and optimisation of dung as a fertiliser.

2.5 Conclusion

Dung degradation is a complex and dynamic process which is driven by numerous abiotic and biotic factors. The environment in which dung is deposited is the significant determining

factor as to how it will degrade when compared to dung biochemical composition. Over time, non-fibre components of dung are removed and as a consequence dung becomes more fibrous. Significant observed differences in dung degradation highlight the impact that could be posed to nutrient cycling within agricultural systems which, when scaled up, may have compounding knock-on effects on productivity. Significant differences between feed and dung types were found across a range of variables. However, the three farmlet's are more alike than they differ and it is reasonable to assume that the differences found would be far greater if sampled across a wide geographical range of beef farms with a variety of management regimes. Therefore, the significant, even though often small, differences found between feed types and dung types highlight the significant role that dung plays within pasture-fed livestock systems. Results support a case for more detailed consideration of nutrient qualities of livestock dung and the role that it can play in productive and sustainable livestock systems. The lack of significant differences in cattle carcass prices provides reassurance that subtle changes in pasture type are unlikely to impact end-product value heavily.

To comprehensively assess grazing livestock systems, particularly with respect to sustainability, it is essential for there to be a greater scientific focus on dung composition and dynamics, especially concerning dung as a nutrient and fertiliser. This work provides insight into how aspects of that may be achieved.

Chapter 3

Anthelmintic impacts - Modelling the impact of targeted anthelmintic treatment of cattle on dung fauna

The primary work of this chapter has been published in a peer-reviewed journal. A copy has been provided in Appendix (8.8).

Cooke, AS, Morgan, ER & Dungait, JA, 2017, 'Modelling the impact of targeted anthelmintic treatment of cattle on dung fauna'. *Environmental Toxicology and Pharmacology.* DOI: 10.1016/j.etap.2017.02.012.

Summary

The insecticidal properties of many anthelmintics pose a risk to dung fauna, through the effects of drug residues in dung on the activity, oviposition, and development of dung-dwelling invertebrates. Reductions of dung fauna can inhibit dung degradation, which may impact biodiversity and nutrient cycling on farms. A simulation model was created to predict the impact of antiparasitic drugs on cattle dung fauna, and calibrated using published data on the dung-breeding fly, *Scathophaga stercoraria*. This model was then tested under different effective dung drug concentrations (EC) and proportions of treated cattle (PT) to determine the impact under different application regimens. EC accounted for 12.9% of the observed variation in *S. stercoraria* population size, while PT accounted for 54.9%. The model outputs indicate that the 'best practice' within veterinary medicine for targeted selective treatments (TST), in order to attenuate selection for drug resistance in parasite populations, will reduce the negative impacts of treatments on dung fauna populations by providing a population refugia. This provides novel evidence for the benefits of TST regimens on local food webs, relative to whole-herd treatments. The model outputs were used to create a risk graph for stakeholders to use to estimate the risk of anthelminthic toxicity to dung fauna.

3.1 Introduction

In

Chapter 2 it was shown how the management of pasture can influence the properties of cattle dung and how it degrades - the observed variations, driven by pat location were most likely facilitated by local biology. However, this is not the only management decision which has the potential to impact dung dynamics. Pharmaceutical, veterinary interventions applied both prophylactically and reactively, are core aspects of farm management decision making, essential for the maintenance and improvement of animal health. Integral to this strategy is the use of anthelmintics. Anthelmintic medicines are widely and routinely administered to

grazing livestock to control gastrointestinal nematodes and other parasites and commonly administered across entire herds in single instances at similar times. Residues of anthelmintics can be found in the dung of the treated animal, and these residues can have insecticidal properties, therefore reducing the biological diversity and activity within the dung. Dung biology is key in the degradation and turnover of nutrients. Dung acts as a vital material for the recycling of nutrients, particularly of N and P, within livestock systems (Lovell and Jarvis, 1996; MacDlarmid and Watkin, 1971; Williams and Haynes, 1995). The release of these nutrients into the environment relies on a range of biotic and abiotic factors, which facilitate the breakdown and incorporation of dung into the soil, where it can then act as a fertiliser. Dung is a rich environment for many invertebrates, fungi, and other microorganisms and soon after it hits the ground, a biological invasion occurs.

3.1.1 Dung biology

Within the dung, biological activity drives a range of essential processes such as the turnover of nutrients into the soil, for use by plants, and to the broader ecosystem food web. Annually, dung beetles alone are estimated to provide £367million of ecosystem services to the UK cattle industry, an equivalent of approximately £40 per animal (Beynon et al., 2015). The biological dynamics within dung are complex, organisms use dung as a food source, as a refuge, or as an opportunity to predate. This creates a dynamic food web which, over the life-span of a dung pat, grows and collapses. No organism exists in isolation within the dung, and the dung community does not exist in isolation from the rest of the environment. For example, dung invertebrates can impact the competition for resources between bacteria and fungi (Lussenhop et al., 1980).

3.1.1.1 Insects

This invasion of insects is relatively orderly and over the course of succession, the food web within that dung increases in complexity (Hafez, 1939; Snowball, 1946; Valiela, 1974). Burrowers are typically the first to invade dung and persist for the lifespan of the dung pat's food web. These are typically burrowing beetles (Superfamily: *Scarabaeoidea*), of which there are approximately 100 species in the UK. These beetles create channels within the dung, aerating it and providing access points for other species, typically predatory beetles, such as rove beetles (Family: *Staphylinidae*). Such beetles are quick to colonise, initially smaller

species, but as the dung food web develops, larger species invade. Dung flies (Family: *Scathophagidae*) are also quick to invade and lay eggs. The dung provides a suitably warm, moist, and nutritious environment for their offspring. The larvae, which are coprophagous (Blanckenhorn et al., 2010), are a vital food source for predatory beetles, but quickly outgrow this vulnerability. As the food web develops further, more and more species invade, such as predatory mites, parasitic hymenopterans, smaller dipterans, hydrophilids, and many more (Valiela, 1974). Such insects are not just part of the dung food web, but the wider food web of the local ecosystem. For example, mature dung flies are predatory, feeding on other dipterans (Blanckenhorn et al., 2010; Cotterell, 1920; Sasaki, 1984) and are themselves the prey of various species of birds and bats. Invertebrate activity in dung may also affect the development of GINs which are inadvertently buried by dung beetles, thereby reducing larval availability on pasture (Bryan, 1976; Fincher, 1973; Sands and Wall, 2017)

3.1.1.2 Earthworms

Earthworms have been shown to significantly contribute to the degradation and disappearance of cattle dung (Barth et al., 1994a; Dickinson et al., 1981; Holter, 1979; Madsen et al., 1990b). Earthworms can be split into three main groups, epigeic, endogeic, and anecic, each of which have unique functions within the soil ecosystem. Epigeic earthworms remain on or near the soil surface, moving horizontally through litter and the uppermost strata. Endogeic earthworms live slightly deeper, although predominantly move on the horizontal plane, and tunnel through the topsoil and upper strata. Anecic earthworms move vertically, creating deep and extensive burrowed networks. The variation in movement between these groups facilitates the incorporation of dung nutrients throughout the soil strata. As a result, earthworm activity has been linked to improved soil quality and plant nutrient uptake (Chaoui et al., 2003; Stork and Eggleton, 1992). In addition, earthworms have been shown to reduce the burden of GIN infections in ruminants, within the same system, through their impact upon GIN larvae in dung, most likely through consumption of GIN eggs (d'Alexis et al., 2009; Waller and Faedo, 1996).

3.1.1.3 Fungi

There is great diversity and richness within fungal communities, and their succession within dung has been well-studied (Harper and Webster, 1964). Many species of coprophilous fungi

are able to break down components such as lignin, which is highly indigestible and can tie up nutrients within cellulose, away from microorganisms (Freer and Detroy, 1982). Fungi can impact GINs within dung through nematophagous activity and the release of mycotoxins (Larsen et al., 1994; Larsen, 2000; Waller, 2006; Waller and Faedo, 1996; Waller and Larsen, 1993).

3.1.1.4 Microorganisms

The significant increases in nutrients, beneath and around dung pats, have been linked to higher soil microbial biomass – an indicator of soil health (Aarons et al., 2009; Ghoshal and Singh, 1995; Helal and Sauerbeck, 1986; Lovell and Jarvis, 1996; Rice et al., 1996). As nutrients from dung are incorporated into the soil, they are utilised and processed by a wide variety of microorganisms. This recycling of nutrients makes them more readily available for uptake by plants, facilitating growth and pasture productivity, whilst simultaneously increasing soil organic matter and carbon (Moe and Wegge, 2008; Williams and Haynes, 1995, 1995).

3.1.1.5 Impact of anthelmintics

Whilst herd health is of paramount importance both ethically and economically; it is also important to consider the use of anthelmintics in the context of the whole farming system. Biological activity is a key contributor to livestock systems, however, this can be negatively affected by the use of anthelmintics, which are not fully metabolized within the host animal and residues of the drug are often excreted in dung (McKellar et al., 1993) and urine (McKellar, 1997). This can, therefore, exert non-target effects on invertebrate fauna which spend part, or all, of their lifecycle in dung (Floate, 1998; Gover and Strong, 1995; Madsen et al., 1990; Sommer et al., 1992; Sutton et al., 2014) and also on soil invertebrates (Scheffczyk et al., 2016). Such effects include inhibited motility, oviposition, emergence, and reduced dung pat colonisation (Floate, 1998; Gover and Strong, 1995; Suarez et al., 2003). As an example, macrocyclic lactones act upon chloride channels in nerve and muscle cells, specific to organisms in the phyla Nematoda and Arthropoda, hence they affect many agricultural parasites and also non-target dung invertebrates. These anthelmintics bind to the glutamate-gated chloride channel, with high affinity, this greatly increases the channel's permeability by permanently 'opening' the gate. The result is a complete inhibition of channel function

leading to paralysis and severe inhibition of function and potentially death (Cheeseman et al., 2001; Köhler, 2001; McCavera et al., 2009; Njue and Prichard, 2004; Wolstenholme and Rogers, 2005).

The detrimental impact of anthelmintics on dung fauna has been observed across a range of species including beetles (Lumaret et al., 1993; O'Hea et al., 2010; Sommer and Nielsen, 1992; Wardhaugh et al., 2001, 1993), earthworms (Diao et al., 2007; Scheffczyk et al., 2016), arthropods (Diao et al., 2007; Scheffczyk et al., 2016), flies, and more (Beynon, 2012; McKellar, 1997; Wall and Strong, 1987). Reductions in the activity of such organisms, as can be caused by anthelmintic residues, consequently reduce the rate at which these nutrients are incorporated in soil and made available to plants, by the slowing of the dung degradation process. (Barth et al., 1993; Lee and Wall, 2006; Madsen et al., 1990b; Wall and Strong, 1987). More rapid incorporation of dung nutrients into the soil is typically beneficial by readily providing valuable resources to plants to invest in growth and structure (Goyal et al., 1993). Quicker degradation also lowers system losses in the case of events such as heavy rainfall which could see nutrients, trapped in undegraded dung, being leached and transported outside of the farm boundary. This negative impact is further compounded by reductions in biodiversity in local ecosystems and the loss of organisms able to mitigate the development of agriculturally significant GINs. In summary, dung plays an integral role in grazing livestock production systems and, as a consequence, factors that influence dung dynamics can have a significant knock-on effect throughout the rest of the system. Effective dung management has the ability to improve system performance in a self-propagating, cyclic manner (Figure 3.1), therefore creating a more sustainable and secure farming system.

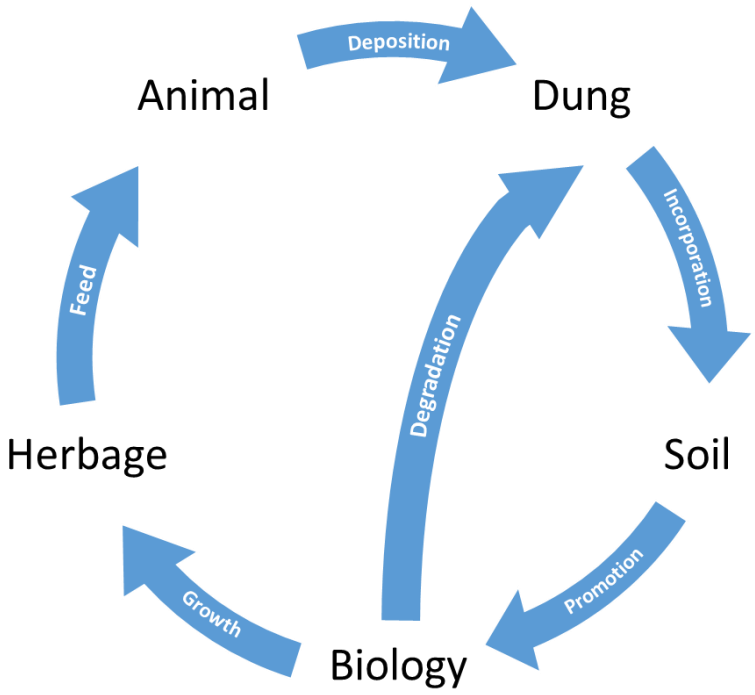


Figure 3.1 – Fundamental structure of the dung cycle and its role within grazing livestock systems.

3.1.2 Targeted selective treatment

The ability to assess and predict the impact of anthelmintics and other routine veterinary medicines on the wider environment is essential for informed drug development and policy in agriculture. In particular, parasite control practices that slow the development of resistance to commonly administered anthelmintics are essential to sustainable livestock production systems, and it is critical that we understand these practices in the context of the total farming system.

In recent years, the mounting resistance of gastrointestinal parasites of domestic livestock to anthelmintic drugs (Geurden et al., 2015; Sutherland and Leathwick, 2011; Waller, 1997, 1994) has led to a shift away from whole-herd treatments, and recommendations for targeted selected treatment (TST) (Charlier et al., 2014). This practice is being promoted for all medicines of infectious diseases. Targeted selective treatment aims to ensure that anthelmintic susceptible genes remain within the parasite population, by providing a refugia of hosts, free from anthelmintics (Besier and Love, 2003; van Wyk et al., 2006). As an indirect effect of TST, the untreated proportion of the herd produces non-toxic dung (i.e. without

anthelmintic residues), which in turn creates refugia of dung for invertebrates to survive. To date, no systematic attempts have been made to evaluate an environmental benefit of TST.

3.1.3 Previous modelling approaches

The scale and complexities of the drug-dung-fauna system are challenging to observe and quantify in vivo and difficult to adequately represent under controlled laboratory conditions. Modelling techniques are a useful alternative to address these issues by allowing the manipulation of a wide range of variables specific to individual field scenarios, and rapid assessments of the potential impacts of new parasite control and other management practices on dung fauna.

Boxall et al. (2007) developed a screening index for assessing the impact of veterinary medicines on dung flies. The index was simple and allowed estimates to be calculated with relatively small amounts of data, allowing rapid screening of multiple drugs. This is a key strength of the index, making it easy to use for non-specialists and non-modellers. The index assessed impact by multiplying three variables: the proportion of cattle treated (p), the proportion of time of faunal contact with dung (q), and dung toxicity (v) to calculate the impact of an anthelmintic treatment (impact = 100.p.q.v). A central assumption was that the three variables are equally weighted. However, there is no justification for this, and such an assumption inadvertently creates a potential mathematical ceiling to drug toxicity. For example, if a hypothetical drug was so toxic that it killed invertebrates on first contact, but the invertebrates were only in contact with it for one day of a 20-day lifecycle, according to the model, mortality would be only 5%. This would evidently not be the case. Whilst such a drug is not in existence or use; this example brings into question the mathematical validity of the model.

Vale and Grant (2002) took a different approach in their development of a model to assess the impact of insecticide-contaminated dung on the abundance and distribution of dung fauna. The model considered a broad and novel range of variables including the response to distinct adverse ecological events on insect lifecycle stages and dung-insect interactions, which aided the understanding of the importance of refugia for the ecology of different species of invertebrates. The methodology was arguably more robust than that of Boxall et al. (2007), primarily achieved through the use of data on invertebrate biology from the

literature, which also makes the model adaptable to different invertebrates, given sufficient data. Whilst the outputs of the work provide significant insight into the topic, no specific tool is produced and, as a consequence, the model is not practical for use by others.

Evaluation of the two aforementioned modelling approaches provides a crucial insight into how new efforts could be formulated in an attempt to harness the strengths of each model whilst limiting the weaknesses. Boxall et al. (2007) created an easy to use model, however, it lacked the use of observational data, whereas for Vale and Grant (2002), the reverse was the case. Therefore, a more effective solution would be to utilise the available invertebrate data in the literature and produce a model which can easily be interpreted by stakeholders and adapted to their needs.

3.1.4 Research objectives

Whilst there has been significant work investigating the impact of anthelmintics on dung fauna, it has overwhelmingly focused on drug toxicity, failing to account for variations in herd coverage, as we would see with TST. Given that TST is the current best-practice veterinary recommendation and is becoming more widely adopted, it is paramount that we now contextualise our knowledge of the environmental impacts of anthelmintics with respect to this industry development.

Herein, the primary objective is to test the hypothesis that the proportion of cattle treated (PT) with anthelmintics has a more significant influence on *Scathophaga stercoraria* populations than the toxicity of drug residue in dung (EC). This is achieved through building on previous theoretical and modelling approaches to create a new modelling approach to simulate the drug-dung-fauna system and to evaluate the potential impacts of antiparasitic drug use in grazed cattle production systems. The model will be used to consider how different treatment regimens administered by farmers, under veterinary advice, for the purpose of livestock health and welfare, have non-target influences on dung invertebrates, and to provide a risk graph to assist stakeholders in selection of the most sustainable options in their livestock production systems.

The secondary objective of the research is to assess the equal weighting of variables, as used by Boxall et al. (2007), by comparing results gained in this work, to the mathematical principles used in their paper. The following hypotheses will be tested:

Hypothesis 1 – Differences in simulated anthelmintic treatment regimes will have a significant impact on populations of modelled dung fauna.

Hypothesis 2 – There is no significant difference between the results gathered from the newly created model and those reported by Boxall et al. (2007).

3.2 Methods

3.2.1 Concept formulation

Both Boxall et al. (2007) and Vale and Grant (2007) developed models with significant pros and cons. The founding principles of this newly proposed model were based on the pros from both of the aforementioned models.

The model was required to have a usable output. Given the complexity and uniqueness of most modelling software, it was decided that the model needed to provide a tangible output that can be utilised without manipulation or specific technical expertise. A risk chart, for different treatment regimes, was decided upon as the most suitable way in which to achieve this.

It was also essential that the model utilised as much observational data, from the literature, as possible. The most abundant and relevant information available was on invertebrate lifecycles. This was to become the foundation from which the model ran. If lifecycles could be successfully modelled, a toxic anthelmintic could then be introduced at a later stage. This would also allow for other invertebrates to potentially be modelled, provided enough published lifecycle data was available.

The final concept was to create an invertebrate lifecycle simulation, using secondary observational data. This would then be used as a model to test different treatment regimes, the results of which would be formatted to create a simple risk chart which would broadly assess a range of treatment regimes. Simultaneously, this would create an easy to interpret and highly visual result, ensuring accessibility of the outcomes across stakeholder groups.

3.2.2 Software selection

There is a wide variety of modelling software available for scientific use, and it was necessary to ensure that the most appropriate software was selected to ensure that the research objective could be achieved. The candidate software were R and NetLogo both of which are non-commercial freeware programs, developed by academic institutions.

R is the most used modelling software within STEM research and uses its own programming language (called R). The programme's appeal is down to the simplicity of the platform which allows users to build and develop models from the ground up and with few restrictions. Widespread usage of R means that an extensive range of packages (pre-programmed R add-ons) exist, which themselves can be built on. In addition, there are numerous active R communities online which can be used as a reference in model development and troubleshooting.

NetLogo is most commonly used for ecological work and spatial modelling and described as a "*multi-agent programmable modelling environment*". This is manifested by a virtual environment in which agents (known as 'turtles') exist and function as prescribed (Figure 3.2). The benefit of this is that each turtle moves and acts as an individual, only interacting with others if prescribed. For example, if a model had 100 sheep, each sheep would be an individual 'turtle'. A key benefit of the NetLogo interface is the ease with which data can be viewed in real time as it is processed, as can the turtles. During programming, this allows for errors or anomalies to be easily spotted. Whilst such a system would be possible in R, that itself would require intensive high-level programming. Therefore, R models typically generate final values at the end of simulations, making it more difficult to identify potential errors during the simulation process. It is for these reasons that NetLogo was chosen in this instance.

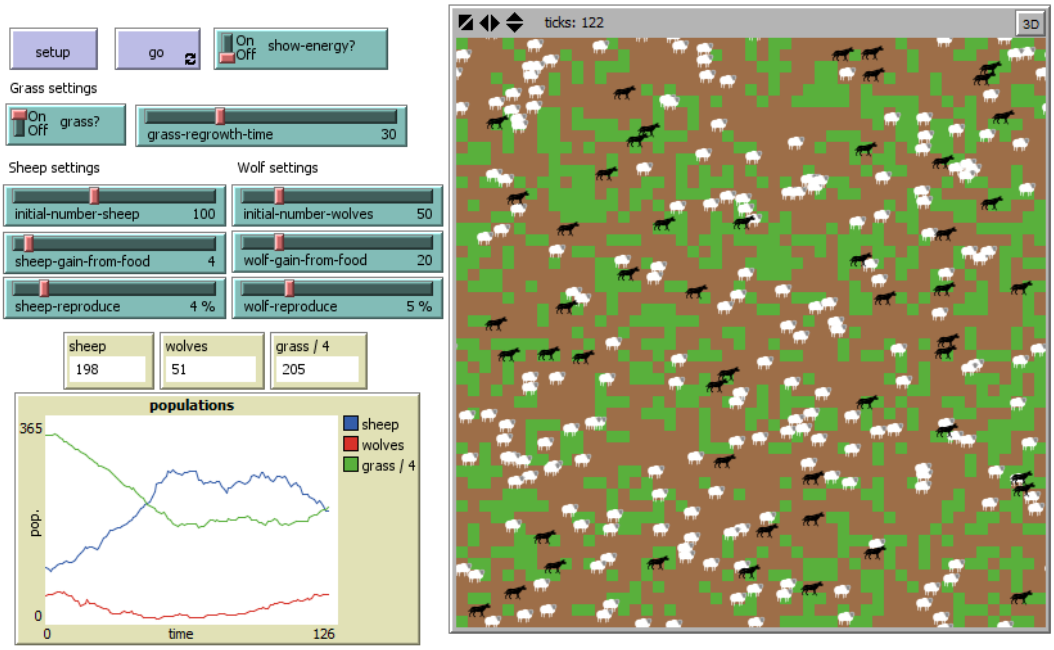


Figure 3.2 - NetLogo interface showing variables (sliders, top left quadrant), real-time data (bottom right), and modelling environment (right). Source: original, using NetLogo library model (Wilensky, 1999).

3.2.3 Model description

A simulation model was created using NetLogo 5.0.4 (Wilensky, 1999) to estimate the impact of a hypothetical anthelmintic that expressed insecticidal properties when excreted in dung by cattle in a grazed field, upon the population of a model dung invertebrate. A 2-dimensional virtual pasture system was created, occupied by a herd of cattle and a population of the model invertebrate. All actions and interactions presented were simulated in hourly time-steps for each individual cattle or invertebrate, as appropriate.

3.2.4 Components

The model simulated the interaction between a model dung invertebrate and cattle defaecation behaviour, and potential for invertebrate survival to be changed by different concentrations of anthelmintic residues in the dung.

The model invertebrate was the yellow dung fly *Scathophaga stercoraria*. The model utilized published data (Table 3.1) to simulate the lifecycle of *S. stercoraria* in a temperate cattle grazing system. *S. stercoraria* is a well-studied dung fauna species, for which detailed information on lifecycle parameters is widely available. The species is highly abundant across the northern hemisphere and some of its lifecycle stages are dependent on dung. *Scathophaga stercoraria* lay their eggs in fresh dung, the hatched larvae then feed on the dung before developing into flying adults.

The model cattle were based on published data on temperate grazing commercial beef and dairy herds (Table 3.1). There were two components to cattle behaviour: (1) defaecation frequency, and (2) randomized movement across a field. The cattle were treated with a hypothetical anthelmintic, or untreated, producing toxic or non-toxic dung, respectively. The proportion of cattle treated (PT) ranged from 0 to 1 in increments of 0.1 and was specified as an independent variable in each simulation.

The rate of defecation by the model cattle and the mean carrying capacity of resulting dung for *S. stercoraria* was based on published data for temperate commercial beef and dairy systems (Table 3.1).

The strength of the toxicity, i.e. Effective Concentration (EC) also ranged from 0 to 1 in increments of 0.1 and was specified as an independent variable in each simulation. The dung became unattractive for *S .stercoraria* regardless of toxicity after a simulated 120 h (= 5 days).

Table 3.1 - Model variables and values used for simulations. Values are taken from observational and manipulative experiments available in the literature. Mean values are fixed constants other than those with a standard deviation (S.D.) which were random variables simulated across a normal distribution by random number generator using NetLogo 5.0.4. Sources: [1.] Blanckenhorn, (1997), [2.] Blanckenhorn et al. (2010), [3.] Römbke et al. (2009), [4.] Martin et al. (2004), [5.] Aland et al. (2002), [6.] Gary et al. (1970), [7.] Oudshoorn et al. (2008), [8.] Sahara et al. (1990), [9.] Villettaz Robichaud et al. (2011), [10.] Floate (1998), [11.] Vale and Grant (2002), [12.] Geiger (2010), [13.] Parker (1970).

Variable [source]	**Value**
Dung fauna (*S. stercoraria*)	
Adult life span (emergence to death)[1]	44 days
Juvenile period (egg to emergence)[2]	22 days
Female:male ratio[1]	1:1
Dung preference[3]	0
Progeny to reach adulthood[4]	10.8 (2.9)
Cattle and dung	
Mean daily defecation rate (pats per day)[5-9]	11.2 (2.4)
Dung attractive period (with drug residue) to *S. stercoraria*[3,10,11]	5 days
Dung attractive period (no drug residue) to *S. stercoraria*[3,10,11]	5 days
Mean dung pat carrying capacity for juveniles[12]	4.3
Season length [13]	6 months
Number of cattle	20

A starting population of 100 individuals of *S. stercoraria*, covering a random distribution of ages within typical life expectancy for the species, were simultaneously introduced to the system. They actively sought out cattle dung in order to produce offspring with no preference for toxic or non-toxic dung. Population fitness responses of the *S. stercoraria* to contact with toxic dung was based on the interaction between specified PT and EC values.

Primary assumptions were:

(i) the model dung from treated animals retained a constant toxicity for 120 h

(ii) there were no sub-lethal effects of the anthelmintics upon *S. stercoraria*

(iii) there were no additional sources of mortality for *S. stercoraria* other than toxicosis or exceedance of lifespan

(iv) the population of *S. stercoraria* is isolated.

No values or weightings of variables model were assumed or given arbitrary values.

3.2.5 Implementation

3.2.5.1 Primary simulations

The model was run 605 times. Each run simulated 4380 h (6 months) using all combinations of 11 PT values and 11 EC values, totalling 121 unique sets of parameter values. There were five repeats of each set, with variable outcomes depending on values simulated from normal distributions: the mean of each set of repeats was used for statistical analyses. The Anderson-Darling normality test was conducted on residuals for the dependent variable of final population size at the end of the simulated period to ensure appropriateness for parametric testing. This was followed by Pearson's correlation analyses of final population size versus PT and EC. Multiple regression analyses were then conducted to attribute how much of the variation in final population size was due to PT and EC, respectively.

3.2.5.2 Secondary simulations

Individual paired simulations was run to evaluate the index created by Boxall et al. (2007). These simulations were performed in pairs in which the product of PT and EC was equal, but the individual values of PT and EC in each pair were not equal. To achieve this the values for PT and EC of pair 1 were switched to form pair 2 (Table 3.2). For the Boxall et al. (2007) model to agree with the presented model, there should be no significant differences between pairs that meet the aforementioned rules. Final population numbers from simulations were then subject to the Paired *t*-test.

Table 3.2 - Values of PT and EC for paired simulations in order to evaluate Boxall et al. (2007) model.

	Group A		Group B	
Pair no.	**EC**	**PT**	**EC**	**PT**
1	0.0	1.0	1.0	0.0
2	0.1	0.9	0.9	0.1
3	0.2	0.8	0.8	0.2
4	0.3	0.7	0.7	0.3
5	0.4	0.6	0.6	0.4

3.3 Results

The distribution of final population sizes across all simulations was non-normal (Anderson-Darling, $p < 0.005$; Figure 3.3). The data shows two distinct groupings based on final population size, one at 0 and the other in the region of 3100 to 4300. This latter group, the 'maximum fitness' group, had a normal distribution ($p = 0.383$). Quartiles for the maximum fitness group were measured as Q0 = 3259, Q1 = 3597, Q2 = 3703, Q3 = 3798, Q4 = 4197. PT and EC combinations that resulted in final populations of < Q0, and therefore outside of this group, were considered as high risk. Combinations that fell between Q0 and Q1 were considered medium risk, and all over combinations resulting in final populations > Q1 were considered low risk (Figure 3.4).

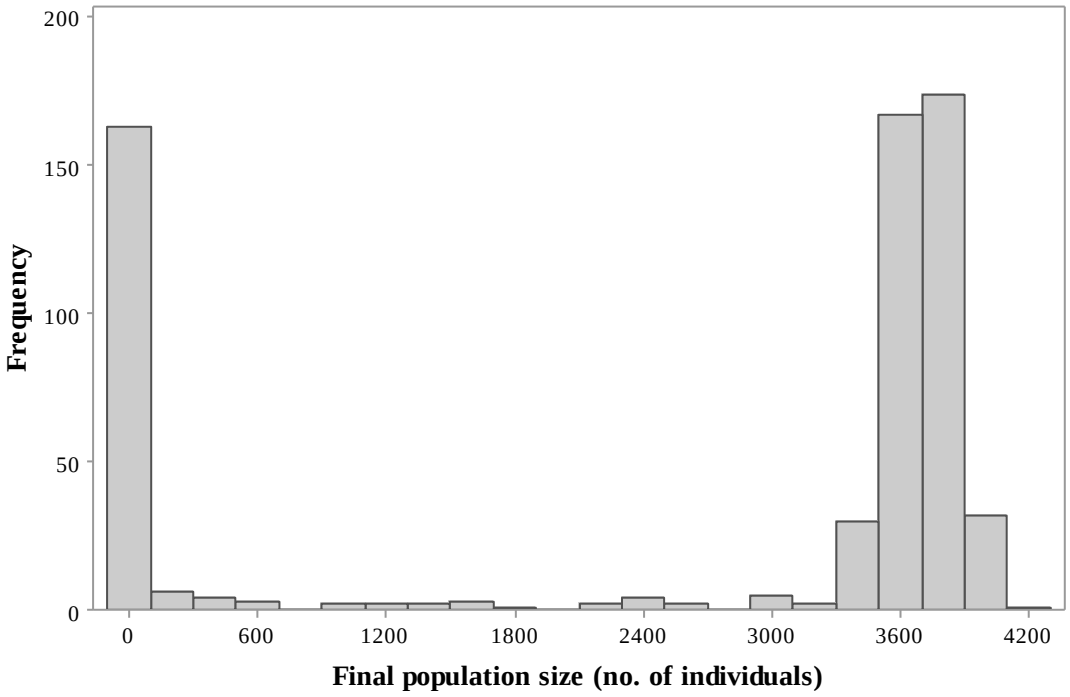


Figure 3.3 - Histogram showing the distribution of final population sizes across all simulations.

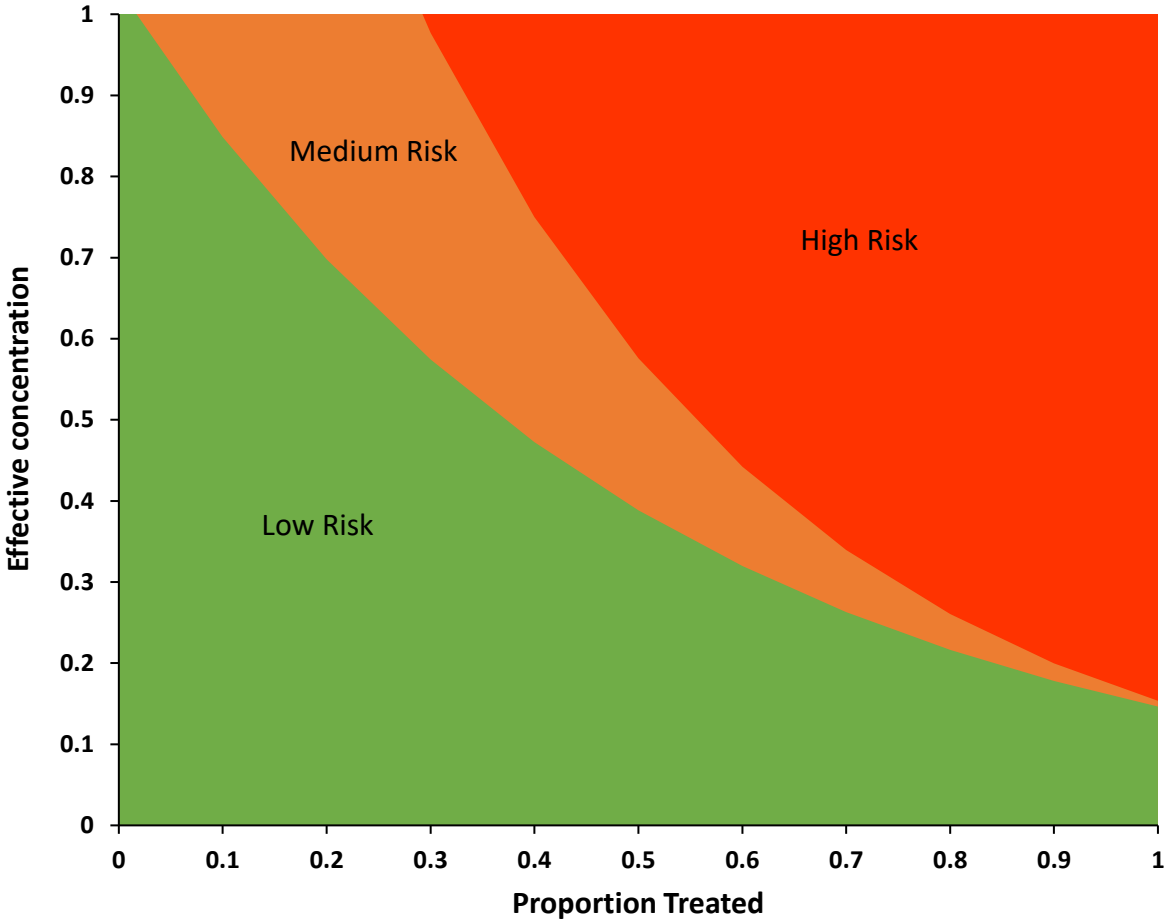


Figure 3.4 – Predicted risk of drug toxicity of different treatment regimes on S.stercoraria, *based on results of all primary simulations.*

In general, incremental increases in PT and EC at low levels had little effect on final population size (= no. of individuals), but a tipping point was reached beyond which the population decreased exponentially (Table 3.3). Rising EC values from 0.0 to 0.5 brought about gradual decreases in final population size; however, as EC exceeded 0.5 its further incremental effect on population size reduced. In contrast, rising PT values of 0.0 to 0.5 had little impact on population sizes, but as PT exceeded 0.5, there was a rapid drop in population size. If 40% or fewer of the herd were treated, risk to populations of *S. stercoraria* was low, regardless of EC.

Table 3.3 - Mean simulated final population size for varying proportions of cattle treated (PT) and effective dung drug concentrations (EC). PT and EC range from 0 to 1.0 in intervals of 0.1, so simulations were conducted for 121 scenarios, representing every PT and EC value combination. Results are plotted here as a heat map, intense red representing a final population of 0 and intense green the greatest final population size (3859), with intermediate values transitioning through yellow.

		Proportion Treated										
		0.0	**0.1**	**0.2**	**0.3**	**0.4**	**0.5**	**0.6**	**0.7**	**0.8**	**0.9**	**1**
Effective Concentration	**0.0**	3678	3676	3701	3763	3645	3739	3770	3696	3590	3642	3608
	0.1	3675	3681	3734	3699	3652	3765	3767	3650	3574	3598	3713
	0.2	3764	3697	3692	3685	3659	3629	3741	3635	3044	1353	338
	0.3	3789	3753	3655	3777	3659	3777	3409	2950	1422	0	0
	0.4	3859	3721	3758	3722	3603	3789	2784	1756	0	0	0
	0.5	3667	3620	3705	3807	3294	3325	2112	0	3	0	0
	0.6	3658	3661	3701	3777	3783	3147	1481	85	0	0	0
	0.7	3738	3786	3816	3723	3755	2902	1385	762	0	0	0
	0.8	3750	3661	3671	3655	3660	3310	1634	744	4	0	0
	0.9	3761	3665	3689	3724	3178	2594	1586	8	0	0	0
	1	3790	3680	3754	3564	3745	2337	0	724	0	0	0

The residuals of the complete data for all experimental runs were normally distributed as tested by Anderson-Darling test (p = 0.281), and thus no transformation was required for parametric analyses. A Pearson's correlation analysis showed that final population size was significantly correlated with PT (-0.694, $p < 0.001$) and EC (-0.336, $p < 0.001$). A subsequent multiple regression calculated the total variance of final population explained by PT and EC together, R^2, to be 67.8% ($p < 0.001$). Further individual regressions showed that PT explained 54.9% ($p < 0.001$) and EC explained 12.9% ($p < 0.001$) of total variance in final population size.

Source of remaining variation came from randomness built into the model, meaning that no two simulations, even repeats, played out identically.

The paired *t*-tests, for the purpose of evaluating Boxall et al. (2007), showed a statistically significant intra-pair difference ($t = 2.43$, $p = 0.023$) and therefore the H_0 was rejected. That is: simulations of which the sum of PT and EC are equal, do not yield equal results.

3.4 Discussion

In this study, a novel simulation was used to test the hypothesis that PT had a more significant impact on the population size of *S. stercoraria* than EC. The outcomes of 605 simulations of 121 pairings of PT and EC indeed indicate that this hypothesis can be accepted. The distribution of data predicted that populations of *S. stercoraria* were generally resilient and can maintain stable numbers up until a tipping point at which population extinction becomes probable without support from immigration. As such, our model builds upon the concept of the screening level index (Boxall et al., 2007), through simulation modelling using published data on key lifecycle parameters that could strongly influence drug-insect interactions. A new approach is proposed, providing a better justified mechanistic framework for impact assessment, which will improve recommendations on the use of veterinary medicines with consideration for livestock dung ecology and wider impacts on the environment.

In addition to the immediate informative value of the results, the model provides a framework that is adaptable to dung-breeding insect species other than *S. stercoraria*. Application to other target species, however, would require further empirical information on the toxicity of various drugs, as faecal residues, on specific fauna. Moreover, lifecycle parameters specific to other species would be required, although the model could also be used to explore parameter space and identify broad characteristics of species that are likely to be vulnerable to anthelmintic residues in dung, and the extent to which these might be attenuated by TST. Currently, data to extend the model to other species is lacking within the literature and therefore observational work would be required to accomplish this. Since the model framework was developed using a bottom-up approach, by creating a model life cycle and then introducing variables on top of it, it lends itself to constructive adaptation and expansion. With sufficient observational data, there is scope for future models, within such a framework, to increase in complexity and realism. Expansion of the model to represent multiple

invertebrates at farm level would enable holistic landscape-scale impact assessments and attenuation strategies. The use of veterinary medicines, with non-target insecticidal properties, is widespread across the developed world and therefore the applicability of observed results may be equally wide. However, the data from the literature that provided the foundation for the model was predominantly derived from studies in temperate regions. Climatic differences between regions in the temperate zone, and regions in the tropics, may have a significant impact on the ecotoxicity of such medicines (Kryger et al., 2005). In addition, the model was based upon a set stocked system and is unlikely to accurately represent the ranch/range rearing of cattle seen across some areas of North America, South America, and Australia. Nevertheless, the model provides a framework for the development of future similar work and with suitable real-world data could be adapted and enhanced to apply to the specific characteristics of different cattle production systems across the world.

3.4.1 Nutrient turnover

The model demonstrates the impact that anthelmintics can have upon dung fauna, in particular, the potentially severe population outcomes that could arise from high levels of anthelmintic treatment. This might explain the significant reductions in dung fauna symptomatic of many grazing livestock systems throughout the UK elsewhere (Schon et al., 2012). Loss of invertebrate activity may impact farm level nutrient turnover through the inhibition of degradative processes, essential for the incorporation of dung into the soil ecosystem. Reduced turnover may ultimately decrease pasture productivity (Goyal et al., 1993), which consequently can limit stocking density and/or increase dependency on costly external nutrient inputs, such as inorganic fertilisers. All these factors impact upon the economic and environmental sustainability of farming systems in the long term.

3.4.2 Targeted selective treatment

Results provide supporting evidence for the ecological benefits of farm management strategies that actively limit the use of anthelmintics and other veterinary medicines. A particular benefit is observed for farms that implement TST strategies that therefore rarely dose entire herds. Cow pats in grazed systems without drug residues may provide a significant refugium of biodiversity, allowing maintenance of populations of coprophagic fauna, e.g. dung beetles and insect larvae, which are important for ecosystem services

including nutrient cycling, carbon cycling, and soil quality. Therefore, TST, as opposed to whole-herd treatments, is recommended in order to reduce the impacts of drug treatment on local ecosystems. Maintenance and enhancement of the local ecosystem can yield potential economic benefits through reducing the need for manual farm inputs (Charlier et al., 2012). However, potential gains in on-farm ecosystems (i.e. improved biological activity and biomass) must be considered in the context of the wider farm system. It is noted that an inadequate TST strategy could reduce animal health, through parasite-driven pathology, and subsequent performance. Therefore animal health should be central to the decision-making process – both the short-term health of the current animals, ensuring that they are not suffering from parasitic diseases, and the long-term health of the herd in terms of anthelmintic resistance and the farm's future capabilities to cope with to parasitic infection.

3.4.3 Future work

Despite the high profile of the global threat of drug resistance, the long-term impacts of drugs, especially antiparasitics with non-target insecticidal properties, on the environment are mostly unknown. The topic is a key area for future work to enable effective assessment and regulation of the use of veterinary medicines, with regards to their impact on all aspects of biodiversity (Adler et al., 2016). Future work in this area should also include economic analysis, in order to balance short-term production gains with longer-term environmental impacts. There is likely to be a utilitarian argument to use veterinary medicines in a more sustainable manner, including the parallel utilization of preventative and non-pharmaceutical methods (Kaplan and Vidyashankar, 2012; Papadopoulos, 2008; Wolstenholme et al., 2004). The emergence of TST is an example of a more efficiently targeted approach to chemical utilization in agricultural systems, which has potential long-term economic benefits, as well as reduced environmental impacts. The current model shows this synergy in quantitative terms for a model insect species and provides a framework for impact assessment and optimization of TST strategies across a broader range of dung fauna, including those of conservation relevance.

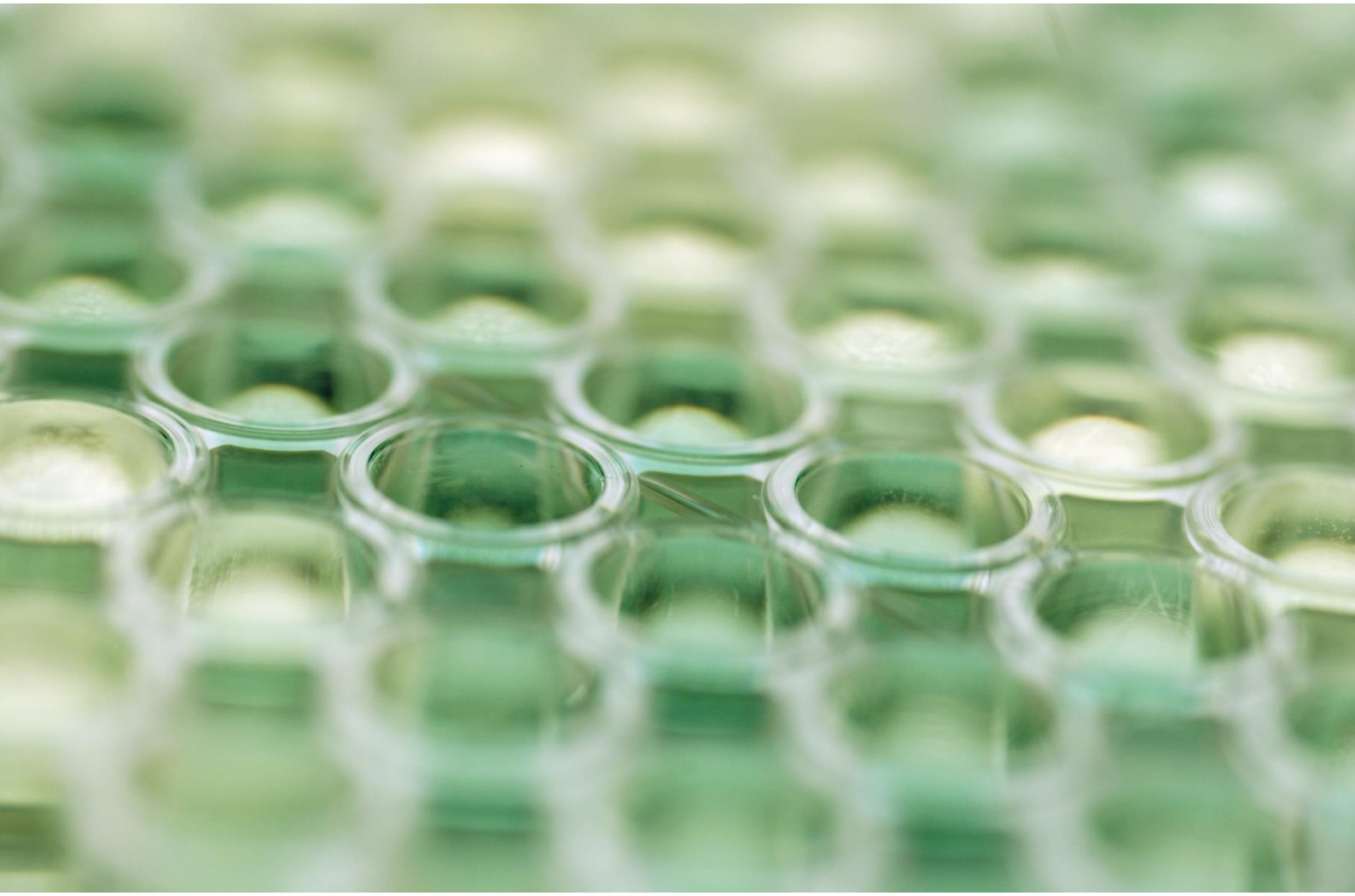
3.5 Conclusion

This research addresses a gap in knowledge of the environmental impact of antiparasitic drug use, by investigating how the herd coverage of anthelmintic treatments may impact dung

fauna, with potential wider implications for local ecology and productivity. The work also outlines a framework of model development which relies solely on published observational data to develop a working lifecycle, negating the need for unnecessary assumptions as seen in prior models in the field. There is significant veterinary support for TST, typically citing short-term benefits to herd health and long-term mitigation of anthelmintic resistance. This work complements such findings by providing supporting evidence for the environmental benefits of TST, compared to whole-herd dosing. This further supports the economic utility of TST as part of a sustainable farming system with respect to animal health, environmental health, and farm economics. Given the increasing body of evidence in support of TST, it is now essential that the tools are available for TST to be effectively and widely implemented. The following chapter addresses this need.

Chapter 4

Faecal immunoassays - The quantification and detection of immuno-markers in cattle faeces



The primary work of this chapter has been published in a peer-reviewed journal. A copy has been provided in Appendix (7.9).

Cooke, AS, Watt, K., Morgan, ER & Dungait, JA, 2018, 'The latest FAD – Faecal antibody detection in cattle. Protocol and results from three UK beef farms naturally infected with gastrointestinal nematodes'. *Parasitology.* DOI: 10.1017/S00311820180000902.

Summary

Gastrointestinal mucosal membranes act as a last line of defence against ingested pathogens attempting to enter the body through the gut wall, such as helminths, bacteria, and viruses. Within livestock production systems, the consequences of diseases caused by such pathogens can be manifested as significant productivity losses through a variety of biological mechanisms. Therefore, gastrointestinal health is central to efficient and productive beef production. Despite this, gastrointestinal immunology is rarely assessed by veterinary professionals due to the lack of availability of adequate methods. The ability to do this efficiently and non-invasively could significantly improve animal health assessments, leading to more informed treatment and intervention. Faecal samples were taken from 114 cattle, across three UK beef farms, with matched blood samples taken from 22 of those animals. A novel faecal supernatant was extracted from faecal samples and serum taken from blood samples. Supernatants and sera were then used in modified enzyme-linked immunosorbent assays (ELISA) for the quantification of immunoglobulins (Ig) IgA, IgG, IgM, and lactoferrin. Non-quantitative ELISAs were also conducted for *Teladorsagia circumcincta* specific IgA, IgG, IgM, and IgE. All assays performed successfully, as determined by reference material and controls. IgA was the predominant antibody in faecal material, generating an antibody profile similar to that of mucosal membranes. Whilst faecal immunomarker levels generally did not correlate with blood levels; they did so for *T. circumcincta* specific IgE in numerous instances. Results support the reasoning that faecal ELISAs can be an effective method to gain representative information about the status of gastrointestinal immunology in cattle and potentially other species. As a non-invasive method, not requiring the animal to be present, the method offers significant ethical and practical advantages to the study of farmed and wild animals, for example, for the monitoring of animals which can be evasive or dangerous. The numerous benefits of the technique support the utilisation of the method as part of animal health assessments.

4.1 Introduction

Prior chapters have highlighted the importance of cattle dung within grazing livestock systems. However, faecal material is not just an agricultural resource for system nutrient cycling and ecology but also holds the potential to provide valuable and detailed information about the health status of an animal. Chapter 3 investigated how targeted selective treatment (TST), in addition to its veterinary benefit, has the potential to mitigate the non-target and toxic impact of anthelmintics and other medicines on dung ecology. An effective TST program has the potential to yield short and long term benefits to animal health and the sustainability of veterinary medicines due to the mitigating effect it has on parasite resistance to anthelmintics (Kenyon et al., 2009; van Wyk et al., 2006). The implementation of an effective TST program requires a body of evidence on individual animal health. To achieve this, veterinarians require a toolbox of simple, efficient, and effective diagnostic tools. As it stands, options are limited, and therefore there is a need for the development of novel diagnostic methods. An effective diagnostic tool is one that meets as many of the below criteria as possible:

- Minimises animal stress
- Non-invasive
- Quantitative and comparable
- High-throughput
- Cost and time efficient
- Facilitates mass sampling
- Allows simple sample storage and transport
- Does not require qualification
- Does not require regulatory licensing
- Provides novel information (or an improvement on existing methods)

Currently, FECs (faecal egg counts) are the most widely utilised method for the diagnosis of GIN infections and are central to livestock health assessments. Their popularity is understandable given their low demand for resources and expertise, meeting many of the aforementioned criteria. However, FECs have a number of critical limitations. FECs quantify the severity of infection by the number of eggs per gram (epg) of faeces, but this is inherently

inaccurate. Firstly, the number of eggs shed does not necessarily relate directly to disease severity. An individual with a healthy and active immune response (particularly if it has developed resistance to the GIN), may be able to tolerate a moderate level of infection without significant pathology or the need for veterinary intervention (Råberg et al., 2007, 2009; Restif and Koella, 2004; Roy and Kirchner, 2000). Furthermore, the shedding of eggs in host faeces is temporally and spatially clumped, meaning that repeat FEC of the same animal, or even sample, may yield significantly different epg. Whilst FECs have a central place in veterinary medicine and animal health assessments; their stand alone value is significantly limited. There is, therefore, a gap in the scientific and veterinary capability to efficiently assess gastrointestinal health and immunology of ruminants, particularly in relation to pathogenic drivers, such as GINs.

Within human medicine, faecal samples are used for the diagnosis of gastrointestinal diseases, through the identification of inflammatory and other markers (Røseth et al., 1992; Tibble et al., 2000). Recent research has provided supporting evidence that ruminant faeces can also be used, in a similar manner, such as for the identification and quantification of antibodies (Watt et al., 2015). The ability to non-invasively assess aspects of animal immunology could open up an entirely new field of diagnostics within veterinary medicine whilst simultaneously yielding significant practical benefits across the wider biological sciences.

4.1.1 Immunomarkers

Immunological responses are vital, yet costly, processes for all organisms (Bonneaud et al., 2003; Råberg et al., 2000). Ruminants are constantly challenged by a host of pathogens which they must actively work to resist. They achieve this through the immune system, which relies on a variety of complex processes and molecules, such as immunoglobulins, to fight off infection and maintain health. In addition to increased health and welfare, healthy animals perform more efficiently and are therefore more profitable (Gerloff et al., 1986; Hawkins, 1993; Schneider et al., 2009; Smith, 1998; Snowder et al., 2006). Improved animal performance has consequential benefits for improving the economic and environmental sustainability of livestock systems.

4.1.1.1 Antibodies

Gastrointestinal mucosal membranes are an important component of mammalian immune systems, providing a protective barrier against pathogens, such as GINs and other helminths (Claerebout and Vercruysse, 2000; Smith et al., 1985). A key feature of mucosal membranes is antibodies/immunoglobulins which directly combat pathogens and other foreign bodies in an attempt to prevent their entry into host tissue. There are five primary isotypes (classes, which differ in structure and function) of immunoglobulins (Ig): IgA, IgD, IgE, IgG, and IgM, which all serve different and unique functions. Each isotype can have numerous subtypes, which play subtly different roles and are generally localised to specific systems or tissues. Due to the different function of each isotype, they each operate in distinct locations and have specific functions. In addition, optimised pathogen-specific antibodies can be biosynthesised locally (i.e. in the gut) (Janoff and Frank, 2010). Antibodies can be quantified by a range of techniques, the most common and fundamental being by ELISA (Enzyme-linked immunosorbent assays; Wide and Porath, 1966), however, more advanced techniques exist, such as protein microarray technology (Templin et al., 2002). Heightened antibody levels are often symptomatic of disease (Dong et al., 2008; Newkirk et al., 2005). However, baseline levels vary between individuals. Over the course of an infection, antibody levels vary greatly and are typically characterised by a primary and secondary response. Therefore antibody levels at the infection site may not necessarily correlate with disease burden.

IgA is locally produced by plasma cells and is transported across epithelial cells where it is released into the external mucous secretions that line the gut (Snoeck et al., 2006). As a result, IgA is the most abundant antibody of mucosal membranes, typically totalling more than all other antibody classes combined (Brandtzaeg, 2013; Golby and Spencer, 2002; Lamm, 1988). The most abundant form of IgA in mucosal membranes is secretory IgA (sIgA), which has an additional secretory component, protecting the molecule from the proteolytic enzymes of the digestive system (Lindh, 1975). The primary role of IgA is to prevent the binding of pathogens and their antigens to epithelial (and other) cell walls (Borén et al., 1993; Mazanec et al., 1993; Williams and Gibbons, 1972), thus preventing their pathogenic function. IgA can limit the length of nematodes, a factor directly related to their fecundity, and is therefore important in the immune response to gastrointestinal nematodes and other parasitic diseases (Stear et al., 1999; Strain et al., 2002). Whilst the full details of this mechanism are unknown there is

evidence suggesting that IgA may facilitate the transport of other immunological molecules (Kaetzel et al., 1991) and that it may activate eosinophils (Decot et al., 2005).

IgD is by far the least studied and most enigmatic isotype. Many questions remain regarding its role and function in immunology, its historical origins, and its unusual interspecies dispersal (Chen and Cerutti, 2010). Despite its ability to bind to many viral and bacterial pathogens, B-cells (antibody excreted lymphocytes) often 'choose' not to secrete IgD during infections. IgD is regularly linked to IgM and may play a complementary role in that relationship. However, the topic is the subject of numerous debates (Chen and Cerutti, 2010; Ohta and Flajnik, 2006).

IgE plays a very specific role in mammalian immunity and is directly associated with immunity to helminth parasites (Erb, 2007; Fitzsimmons et al., 2007; Jarrett and Bazin, 1974; Pfister et al., 1983; Thatcher et al., 1989; Watanabe et al., 2005), as well as to protozoan pathogens (Duarte et al., 2007), and allergens (Gould et al., 2003). During a helminth infection, serum IgE levels can increase by 100x and drop rapidly after infection has cleared (Jarrett and Bazin, 1974). IgE plays a diverse role and has been found to, directly and indirectly, inhibit helminths. The antibody has been linked to the degranulation and stimulation of mast cells and basophils (Rosenwasser and Boyce, 2003; Stone et al., 2010), physical damage to parasites, and the blocking of parasites to epithelial cells. Whilst mucosal membranes are typically associated with IgA and IgM, up to 99% of local IgE can move into the intestines during a helminth infection (Bell, 1996). IgE is, therefore, a significant antibody in the study of veterinary parasitology.

IgG is the most abundant antibody in blood but is also found at mucosal membranes in lower quantities. The antibody has the ability to actively migrate across mucosal membranes into the intestine and is highly abundant in colonic fluid. The primary role of IgG is opsonisation (binding of opsonin to pathogen membranes to attract phagocytes) of pathogens to facilitate phagocytosis (Groux and Gysin, 1990; Koval et al., 1998). The effect of IgG on parasitic nematodes is indirect through its impact on symbiotic bacteria within nematodes, such as *Wolbachia* sp. (Bazzocchi et al., 2000; De Veer et al., 2007; Morchón et al., 2004; Punkosdy et al., 2001; Simón et al., 2003).

IgM is the largest mammalian antibody and is released early in infections as a basic initial response. IgM is produced by B cells without the need for antigenic stimulation, as is the case for other antibodies, ensuring it is always available and ready to respond to pathogens. Upon such a response IgM can readily activate the complement cascade (molecule for molecule, approximately 1000x more effectively than IgG; Cooper, 1985) and therefore enhance the ability of antibodies and phagocytes (Charles et al., 2001). IgM deficiency has been linked to a reduced ability to clear parasitic nematode infections (Rajan et al., 2005). Whilst the mechanism is not entirely clear, IgM readily reacts with surface antigens of pathogens, causing impairment. IgM is, therefore, a vital component of the immune system with the ability to initiate a range of immunological responses necessary for the maintenance of animal health.

4.1.1.2 Lactoferrin

Lactoferrin is a complex and multi-functional transferrin protein present in mammalian immune systems. For diagnostic purposes, lactoferrin acts as a faecal inflammatory marker and within human medicine is used to assess intestinal health and diagnose conditions such as inflammatory bowel diseases and Crohn's disease (Gisbert et al., 2009; Lamb and Mansfield, 2011; Lundberg et al., 2005; Tibble et al., 2000). Intestinal damage is a symptomatic pathological sign of chronic and severe GIN infections. This, in turn, can lead to inflammation for which lactoferrin may be a marker. Commercial ELISA kits are available for the quantification of lactoferrin in the milk of dairy cows, for the purpose of quality control and animal health assessment. Immune responses in GIN infections are typically localised (Stear et al., 1999) meaning that the response to GIN infection predominantly takes place in the mucosal lining of the intestines, making faeces a suitable material in which to attempt to detect and quantify lactoferrin.

4.1.2 ELISA

Enzyme-linked immunosorbent assays (ELISA) are commonly used techniques in molecular immunology for the detection and or quantification antibodies, antigens, and other molecules biological samples (Engvall and Perlmann, 1971, 1972; Wide and Porath, 1966). ELISAs work by capturing a target molecule using complementary antibodies (Figure 4.1). The capture antibodies are typically derived from rabbits or mice which have been artificially exposed to

the target molecules and therefore have produced an immunological response of specific antibodies to that target. ELISAs are now routinely used worldwide and have formed the basis of numerous molecular techniques that have revolutionised the way we investigate immunology and disease. ELISAs are generally replicable, use only a small amount of sample, and allow for a high throughput (potentially thousands of samples per day) at modest cost.

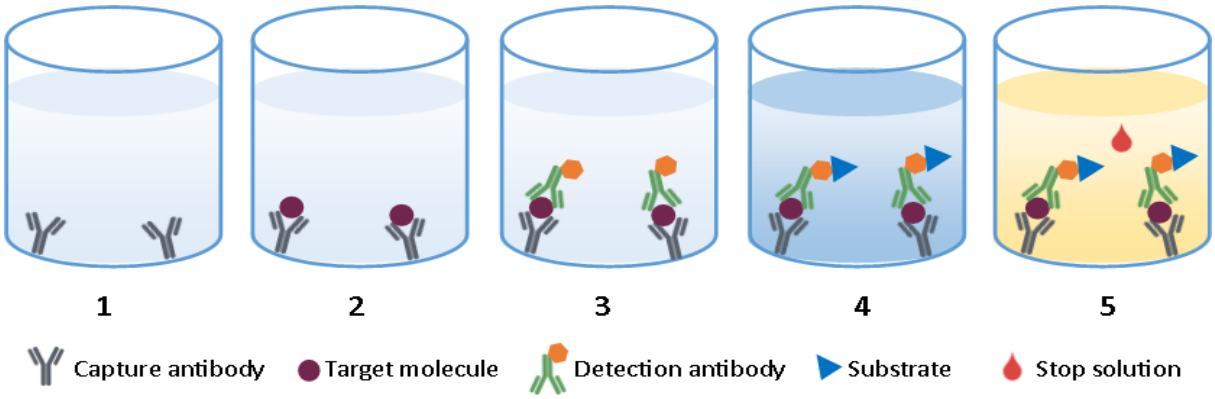


Figure 4.1 – Main steps of a sandwich ELISA. (1) Capture antibody is added and binds to the plate well over night. (2) Sample is added to the well and molecules complementary to the capture antibody, the target molecules, are bound. (3) A detection antibody, conjugated with horseradish peroxidase (HRP) is added and binds to target molecules which were previously bound in step 2. (4) A substrate is added which reacts, facilitated by HRP, to produce diimine, a blue coloured substance. (5) A stop solution is added which significantly alters pH, inhibiting further reactions and producing a yellow colour. As each step sequentially builds upon the prior, the intensity of the yellow colour change is proportional to the concentration of the target molecules, as captured in step 2. Source: original image.

Within the livestock industry, ELISAs and derived technologies are utilised for two distinct purposes: the first is quality and safety control of product, destined for human consumption, the second is to guide production interventions through the monitoring of animal health. Whilst blood is often used, veterinary driven ELISAs are most routinely used within the dairy industry where they can be used for the assessment and diagnosis of a range of diseases using milk samples (Aulakh et al., 2008; Hardin and Thorne, 1996; Kloosterman et al., 1996; Nielsen et al., 2000; Schares et al., 2004; Shafee et al., 2011). Although less universal, within the beef sector, the same technology has also been utilised for meat juice analysis. Charlier et al. (2009) aimed to develop a non-invasive method for the diagnosis of the parasitic trematode *Fasciola hepatica* (liver fluke) and the parasitic nematode *Ostertagia ostertagi* (brown stomach worm) in cattle. Given that sampling was taken post-mortem at the abattoir, the benefit of a non-invasive procedure was for practicality and had no direct bearing on animal

welfare. The study found that ELISA derived antibody concentrations from serum and meat juice samples correlated 90-100%. Meat juice ELISAs were also found to be significantly more accurate in the detection of *F. hepatica* when compared to more labour demanding liver biopsies. Similarly, Cornelissen et al. (1997) evaluated the use of ELISAs for the diagnosis of the parasitic nematode *Dictyocaulus vivparus* (lungworm) in cattle. Results from the original research led to ELISAs being used routinely in all Animal Health Services in the Netherlands, notably replacing Baermann's faecal extractions, which required soaking of faecal samples and subsequent gravimetric recovery of larvae which have migrated from the faeces. This increased the throughput of analyses whilst also increasing their reliability. These examples highlight the potential and relatively unharnessed capabilities of molecular techniques within veterinary medicine. The ability for ELISAs to effectively utilise blood, milk and meat juices suggest that the technique can widely be applied across a range of biological materials. Successes, as outlined, can pave the way for increasing efficiency, welfare, and productivity of livestock industries. Although liquid samples are required, there is the potential for less soluble materials to be suspended in inert liquids, increasing the range of material it is possible to analyse.

Whilst ELISAs are typically conducted on plasma, sera, or milk samples, a small number of studies have utilised animal faecal samples (Duménigo et al., 1996; Peters et al., 2004; Watt et al., 2015; Wedrychowicz et al., 1985), but the area is relatively unstudied. ELISAs on animal faecal material are therefore not well established in veterinary medicine, however, are commonplace in human medicine for the diagnosis of gastrointestinal disease through the quantification of proteins such as lactoferrin and calprotectin, which are present at mucosal membrane surfaces (Kok et al., 2012; Lamb and Mansfield, 2011; Lundberg et al., 2005; Malícková et al., 2008; Mirsepasi-Lauridsen et al., 2016; Schoepfer et al., 2007). Immunological results from faecal material are more likely to be representative of gastrointestinal mucosal membranes than other material such as plasma, due to the physiologically localised nature of immunity (Lamm, 1988; Wennerås et al., 1999), therefore providing novel information about animal health which, if used in tandem with other methodologies, could help to provided more rounded and comprehensive assessments.

Watt et al. (2015) successfully adapted commercially available ELISA kits and components for use on ovine faecal samples collected in the field. ELISAs were also performed to detect

antibodies specifically developed in response to infections of the GIN *Teladorsagia circumcincta*. Successful assays were achieved by replacing the typical sample type (of blood serum or plasma), for a faecal supernatant, formed by mixing faeces with a protease inhibitor and centrifuging to produce a supernatant. The work represented a step towards a viable and novel addition to the toolkit available for animal health diagnostics. In addition to the novel information that the protocol provides, it is also logistically practical and avoids the need for invasive procedures on the animal, which can be time-consuming, stressful (to both the animal and researcher), dangerous, and often impossible. Furthermore, invasive procedures are more heavily regulated than non-invasive ones and require specific training, whether conducted for the purposes of science (*Animals (Scientific Procedures) Act*, 1986) or veterinary medicine (*Veterinary Surgeons Act*, 1966). Due to the fact that the physical and biochemical composition of cattle and sheep dung may vary, a protocol for a faecal supernatant and subsequent ELISAs, designed for sheep faeces, needs to be tested and adapted for cattle faeces.

Bovine lactoferrin ELISA components (capture antibodies, detection antibodies, and reference materials) and kits are typically developed and orientated towards the dairy industry and milk samples, although usually are claimed to work on serum and plasma too. Given the high consumption levels of cow's milk and associated products in the developed world, there is consequently a range of bovine ELISA products available. The extent of choice for other ruminants is not so broad, whilst blood antibody ELISAs are readily available for sheep and deer, lactoferrin is not. Therefore, non-bovine lactoferrin ELISA components are extremely expensive as they must be made to order. Due to the similarity in lactoferrin isotypes across ruminants, it has been observed that bovine-specific ELISAs are able to recognise lactoferrin from all ruminants (Conesa et al., 2008; Shimazaki et al., 1991). This poses the possibility that a bovine lactoferrin ELISA could be successfully applied to other ruminant species, however, the exact extent of cross-reactivity is unknown.

4.1.3 Potential uses

In addition to general annual health assessment, the ability to detect and quantify immunomarkers in faeces has the potential to feed into specific strategies for the prevention and mitigation of gastrointestinal parasites and other prevalent and impactful diseases within

the livestock industry. Increasing the efficiency of diagnostic techniques has the potential to facilitate positive changes in productivity, welfare, profitability, and sustainability. Enabling more effective health monitoring can improve animal health by highlighting problems early on, providing longitudinal data, and by informing veterinarians. The consequence of improved health is improved animal performance with regards to feed conversion and growth. In turn, this improves the utilisation of forage meaning that more outputs (meat, dairy, and offspring) are produced from reduced inputs (forages, fertilisers, land area), therefore improving overall profitability and economical and environmental sustainability.

4.1.3.1 Targeted selective treatment

A key area of opportunity for the utilisation of faecal immunomarker detection is within targeted selective treatment (TST) regimes, which is the recommended practice for the pharmaceutical control of GINs and many other helminths (Charlier et al., 2014; Cooke et al., 2017; van Wyk et al., 2006). TST has the potential to yield long-term benefits to herd health, in part, due to a mitigating effect on the selection for anthelmintic resistanct parasites (Charlier et al., 2014; Gaba et al., 2010). Central to TST strategies is the need for novel and improved tools to effectively implement comprehensive animal health assessments, used to assess parasite burden, anthelmintic efficacies, and ultimately select individuals for treatment (Bentounsi et al., 2012). This screening typically involves qualitative assessment of gross health indicators, such as weight gain, conformation, body condition, and eye colouration (i.e. Faffa Malan Chart (FAMACHA) anaemia scoring), that are non-specific indicators of health. Faecal egg counts (FECs) can also provide relatively quick and simple diagnoses, including species level pathogen identification. The downside of FEC techniques is that egg counts vary temporally and are spatially clumped (Engels et al., 1997; Gasbarre et al., 1996) and are, therefore, not necessarily indicative of parasite burden, and less so of pathology. Consequently, the advancement of TST requires the development of new, high-throughput, diagnostics for rapid assessment of physiological and immunological parameters of animal health – specifically in relation to GINs. Faecal immunomarker quantification, using ELISA techniques, has the potential to become an important part of this tool-kit of techniques, allowing for more detailed and comprehensive evaluations of animal health, therefore enhancing current TST strategies.

4.1.3.2 Selective breeding for GIN resistance

Resistance to GINs is predominantly driven by host genetics and acquired immune responses (Stear et al., 1999). This means that resistance can be selected for during the commercial breeding process. However, this is not common practice, with anthelmintic treatment being routinely favoured by many veterinarians and farmers. In contrast, within wild populations, this amounts to a selection pressure in favour of GIN resistant genes due to the reduced reproductive success of non-resistant hosts. In such populations, where manufactured anthelmintics are not linked, individuals are much more likely to be able to tolerate low or moderate GIN levels without significant impacts upon health (Råberg et al., 2009; Roy and Kirchner, 2000). Increased susceptibility of domestic ruminants to GIN increases the likelihood of pathology up until the point of treatment. The shedding of helminth eggs is typically over-dispersed within host populations, with a small number of individuals shedding the majority of eggs. Removal of these individuals, for the purpose of selective breeding, holds the potential as an effective strategy for the control of GIN and other faecally transmitted helminths (Bisset and Morris, 1996; Gasbarre et al., 2001; Sréter et al., 1994; Stear et al., 2001; Stear and Murray, 1994). Bisset and Morris (1996) make the point that resilience, i.e. the ability to cope with infection, without necessarily clearing it, is also important in combination with selection for resistance. In order to selectively breed animals for these traits, a breadth of information is needed, namely, parasite burdens, host immune response, and genetic drivers of host tolerance. Parasite burdens can be alluded to using FEC techniques, and genome sequencing of parasites is increasingly common; the missing component of such an assessment is, therefore, a measure of host immune responses.

4.1.4 Research objectives

The primary objective of the research discussed in this chapter is to assess if bovine faecal supernatant can be used in ELISAs for the detection and/or quantification of antibodies and lactoferrin.

Hypothesis 1 – Antibodies and lactoferrin can be quantified, via ELISA, in bovine faecal supernatant.

Acceptance of hypothesis 1 would lead to an array of secondary hypotheses, tested to deepen the understanding of the protocols.

Hypothesis 2 – IgA is the most predominant antibody in bovine faecal supernatant.

Hypothesis 3 – Ovine anti-*T. circumcincta* antibodies can be used for the detection of bovine *T. circumcincta* specific antibodies, by ELISA.

Hypothesis 4 – Antibody and lactoferrin levels correlate to one another in faecal supernatant and serum samples.

Hypothesis 5 – Antibodies and lactoferrin concentrations in faecal supernatant do not correlate to those in serum.

Hypothesis 6 – Faecal supernatant lactoferrin concentrations will be significantly different between ruminant species (cattle, sheep, and deer).

Hypothesis 7 – The optical density of faecal supernatants does not correlate with lactoferrin concentration.

4.2 Methods

4.2.1 Sample collection and processing

4.2.1.1 Sample population

Faecal samples were taken from cattle from three UK beef farms.

Farm #1 was at Rothamsted Research's North Wyke Farm Platform, in Devon. The Farm Platform has three non-organic, pasture-fed beef herds, under typically managed rotation. Each herd is similar, however, grazed on distinct pasture systems which vary in their botanical composition. An initial sampling on 10/11/2016 collected 45 faecal samples and the second sampling on 07/02/2016 collected 18 faecal samples, six of which were from animals sampled the first time around. Both sampling instances occurred during housing when animals were on a silage diet.

Farm #2 was a pasture fed beef farm in Hertfordshire. Animals were mob-grazed with movement approximately every three days. Sampling occurred once, on 02/02/2017, during housing, and resulted in 30 faecal and blood samples being taken from 21 animals. The farm was organic (Soil Association certified) and no anthelmintic treatment had been administered that season.

Farm #3 was a pasture fed beef farm in Angus. Cattle were mob-grazed with three daily movements. Sampling occurred once, on 07/12/2017, and resulted in the collection of faecal samples from 30 animals. Animals grazed year round with no housing time. The farm was organic (Soil Association certified) and no anthelmintic treatment had been administered that season.

For the lactoferrin analysis, the opportunity arose to incorporate a number of faecal and blood samples from sheep and deer. One hundred and thirty-three faecal samples and seven blood samples were included from 94 wild sheep. A further 42 faecal samples from 41 semi-wild sheep (commercial animals which are allowed to roam wild most of the year) were also included. One hundred and twenty-six faecal samples were included from 121 semi-wild deer. A blood sample was also included from a semi-wild deer of the same population, which was found recently dead before sampling. None of these additional animals had been recently subjected to any veterinary medicine or intervention.

4.2.1.2 Serum

Tail venepuncture was conducted, by a veterinarian, on live cattle from 22 individuals on Farm #2, to withdraw blood. Blood samples were only collected from animals for which matched dung samples were available, and blood and faecal samples were taken on the same day. Bloods were drawn, by sterile syringe, into labelled 10ml BD Vacutainers® and rested for > 30 min to allow for clotting, then centrifuged at 2500rpm/1056 x g (Sorvall SLA-3000 rotor in a Sorvall RC-5B centrifuge) for 15 min and the supernatant serum withdrawn, using sterile pipette tips, into 1.5ml microcentrifuge tubes (Thermo Scientific™ 3451). Serum samples were immediately stored at -20°C until analysis.

4.2.1.3 Faecal supernatant

Fresh dung was collected upon deposition by the individual. Dung was collected using clean polystyrene spoons, one per sample. Dung was homogenised as thoroughly as possible before collection, with care taken so as not to mix in foreign matter, such as other dung and hay. Collected dung samples were transferred to sterile polystyrene screw-top pots. During sampling the samples were stored in a cool box, after which they were stored at -20° until being processed.

A dung supernatant was obtained by the dilution of cattle dung with a protease inhibitor. In order to create the supernatant, dung samples were allowed to defrost at room temperature for 3 hrs. Defrosted dung samples were thoroughly mixed using sterile inoculating needles (Camlab 1171525). 2-4 g of dung was then transferred to a sterile beaker and mixed with a protease inhibitor (Roche cOmplete™, EDTA-free Protease Inhibitor Cocktail) at a recorded ratio of between 1:1 and 1:2 (w/v). The resulting mixture was homogenised using sterile inoculating needles and then transferred to sterile 10 ml centrifuge tubes (Nalgene™ Oak Ridge High-Speed PPCO) and rested on ice for >10mins, until centrifugation. Samples were centrifuged at 3-6°C and 8400rpm/12000 x g (Sorvall SLA-3000 rotor in a Sorvall RC-5B centrifuge) for 5 min. The supernatant was then pipetted, using sterile pipette tips, into 1.5 ml microcentrifuge tubes (Thermo Scientific™ 3451). Supernatants were immediately stored at -20°C until analysis.

Three negative control blanks for the supernatant diluent, comprised of 100% protease inhibitor cocktail, were created. Each blank came from a different batch of inhibitor cocktail and was prepared separately.

4.2.2 Antibody ELISA

Seven, bovine-specific, ELISAs were conducted. Total IgA, IgG, and IgM ELISAs were conducted using bovine-specific commercial reagents from Bethyl Laboratories Inc. (Texas, United States) and a reference serum, according to the manufacturer protocol. A further three ELISAs were conducted using *T. circumcincta* antigen, measuring the responses of bovine-specific IgA, IgG, and IgM to the antigen. There are no bovine specific IgE components, so a fourth assay was completed using a sheep IgE ELISA. Given the cross-reactivity of ovine and bovine antibodies and the range of GINs which are able to infect both species, there was considered to be a distinct possibility that *T. circumcincta* assays could work on bovine samples. These latter ELISAs were conducted under the same protocol as the commercial ELISAs with the alteration that the commercial capture antibody was replaced with a *T. circumcincta* antigen, as per Watt et al. (2015). No IgD antibodies were readily available for inclusion.

Each ELISA was conducted on all 114 faecal supernatants and 22 serum samples. With each of the Total Ig plates containing a 10 point dilution series of reference material and two or more blanks of TBST (Tris-buffered saline with Tween20 at 0.05%), representing the sample

diluent. Three 'faecal blanks' were also included in each assay: faecal blanks consisted of pure protease inhibitor. The *T. circumcincta* assays do not have a reference serum available, so had a known positive sample included twice (sourced from Watt et al., 2015), which showed the assay worked on that day. The positive control was serum from sheep that had been trickle infected with *T. circumcincta* and had confirmed antigens against L3 *T. circumcincta*, as per Watt et al. (2015).

4.2.2.1 Sample dilution

Supernatant and sera had to be diluted to ensure that ODs were within the detection limits outlined by top and bottom plateaus of their relative sigmoidal curves. Samples were serially diluted and six concentrations (later narrowed down to three) taken forward for use in assays, for each antibody, one dilution was chosen across all samples as the one to derive results from.

4.2.2.2 Laboratory Procedure

96-well plates (Nun-Immuno™ MicroWell™ MaxiSorp™) were coated with 50μl of the appropriate rabbit anti-bovine antibody, diluted to 2 μg ml^{-1} in 0.06M carbonate buffer. For the *T. circumcincta* assays the coat was *T. circumcincta* L3 somatic antigen at 2 μg ml^{-1} in 0.06M carbonate buffer. Plates were then covered in clingfilm, and stored for 1-3 days at 4°C prior to use. Plates were removed from the refrigerator and washed 3x in TBST. Meanwhile, samples were defrosted at room temperature (approx. 1 hr) and then serially diluted in 2 ml deep-well plates. 50 μl of the appropriate sample dilutions were pipetted into the relative wells on the plate. TBST was used as the ELISA sample dilution negative control, Protease inhibitor was used as the faecal sample diluent negative control, bovine reference serum was used as both a plate positive control and, with a dilution curve, to work out antibody concentration in the sample, on the Total Ig assays only. A known positive sheep sample was used as a plate positive control on the four *T. circumcincta* assays. Plates were then covered in cling-film and incubated for 1hr at 37.5°C.

Plates were removed from the incubator and washed 5x in TBST. 50 μl of the appropriate rabbit anti-bovine HRP conjugated antibody was added to each plate (excluding for the *T. circumcincta* IgE assay). No direct HRP-conjugated antibody was available for the *T. circumcincta* IgE assay and instead 50 μl of mouse anti-ovine IgE (monoclonal IgG1) at 10 μl

ml^{-1} with TBST was added. *T. circumcincta* IgE plates were then incubated for 1hr at 37.5°C, washed 5x with TBST and then 50μl of goat anti-mouse IgG1- HRP detection, at 0.125 μg ml^{-1} with TBST, was added. All plates were then covered in cling-film and incubated for 1hr at 37.5°C.

After incubation plates were washed 5x in TBST. 100μl of TMB substrate (KPL SureBlue™ TMB Microwell Peroxidase Substrate – single component) was added to each well, plates were then incubated, in darkness, for 5 minutes at 37.5°C. Plates were removed from the incubator and 100 μl of the stop solution, 1.0M HCl, was added to each well (the addition of HCl inhibits enzyme activity and changes the wells from blue to yellow). Plates were immediately read by a plate-reader at 450 nm, providing the optical density (OD) for each well.

4.2.2.3 Interpolation and adjustment

For each total antibody assay, a 10-point dilution series was plotted as a sigmoidal curve of OD and reference serum antibody concentration. Optical densities were interpolated onto this curve to generate an antibody concentration for each sample of faecal supernatant and serum. These concentrations were then adjusted to account for two instances of *in vitro* sample dilution which occurred initially when faecal supernatants were formed and again during serial dilutions. This generated the final concentration of antibody in each original (unprocessed) sample.

Due to the lack of available reference material for *T. circumcincta*-specific antibodies, it was not possible to interpolate results to generate an exact concentration. Instead a relative and arbitrary scale was created, using the positive control, to allow for simple comparison of samples relative to one another. The value given to each sample was derived from Equation 4.1. As per total antibody assays, results were then adjusted to account for *in vitro* dilution. In the event that negative values were obtained (i.e. if sample OD was less than TBST OD), values were converted to zero.

$$x = \frac{sample\ OD - TBST\ OD}{positive\ control\ OD - TBST\ OD}$$

Equation 4.1 - Formula used to generate a relative and arbitrary scale for T. circumcincta *antibody levels.*

4.2.2.4 Validation

Reference material was essential to confirm the validity of assays and to quantify antibody levels. Total IgA, IgG, and IgM reference material was present on each plate of that antibody type. Reference material stock concentrations were: 0.11, 24.0, and 1.8 μg μl^{-1}, respectively. Twenty-six dilutions of reference materials were formed using halving serial dilutions. The initial dilution wash 80 μl of reference material with 920μl of TBST. 700 μl of that solution was then withdrawn and added to 700 μl of TBST and the process repeated to form a series of up to 26 dilutions, of which 10 were chosen for each assay. Chosen dilutions were based upon past experience of similar assays, which were then tested to ensure suitability. Before experimental assays were conducted, plates were run with the specified dilutions of reference materials to confirm that the generated curves were suitable and within the detection limits of the assay and plate reader, each assay was repeated five times and plates included two blanks of TBST. No IgE reference material was available and, therefore, IgE could not be quantified, only measured in relation to other samples of the same type, however, known IgE positive serum samples were available and used to confirm that the assay worked.

4.2.3 Lactoferrin ELISA

ELISAs were conducted using a commercially available bovine lactoferrin ELISA set (Bethyl Laboratories Inc., E10-126), which is produced primarily for use on bovine milk samples.

The plate coat was made by mixing affinity purified antibody (Bethly Laboratories Inc. A10-126A) with carbonate buffer at a ratio of 1:100 (v:v). 100 μl of the formed coat was added to each well and plates were covered with clingfilm and incubated at 20°C for 1 hr.

Plates were washed 5x with TBST (Tris-buffer saline with 0.05% Tween™ 20), by an automated plate washer. 200 μl of TBST was added to each well as a blocking solution and plates were covered in clingfilm and incubated at 20°C for 30 min.

Plates were washed 5x and 100 μl of sample was added to each well (except blanks). Plates were covered in clingfilm and incubated at 20°C for 1 hr.

Plates were washed 5x. 100 µl of HRP detection (0.5% with carbonate buffer) antibody was added to each well and plates were covered in clingfilm and incubated at 20°C for 1 hr.

Plates were washed 5x. 100 µl of enzyme substrate (SureBlue™ TMB Microwell Peroxidase Substrate Kit) was added to each well and plates were placed in opaque boxes and incubated at 20°C for 15mins. 100µl of stop solution, 0.18m H_2SO_4, was added to each well and plates were immediately read for optical density at 450nm by a plate reader.

4.2.3.1 Assay refinement

A test plate was conducted to determine the optimum concentration of faecal supernatants and serum/plasmas (diluted with TBST) in order to achieve ODs within the detection limits of the plate reader and to be able to observe variation in the datasets. The optimum dilution was qualitatively determined as the dilution at which no notable plateauing or data clustering had begun, which can both be features of more dilute samples. If two dilutions were relatively even on this metric, the less concentrated was chosen in order to ensure capture of lactoferrin samples higher than those on the trial plates and to preserve sample. Test plates were conducted on 63 cattle faecal samples at concentrations of 1/1, 1/2, 1/8, and 1/32 and on 25 cattle serum samples at concentrations of 1/5, 1/10, 1/20, and 1/40. This stage was also used to validate the assay itself, through the yielding of positive results and associated curves.

4.2.3.2 Interpolation and adjustment

Each plate contained a 7 point dilution series of bovine lactoferrin reference material, with known concentrations of lactoferrin, along with a TBST blank. Using GraphPad Prism 6.01, sigmoidal curves were plotted for each reference material dilution series. OD values for samples were then interpolated onto these curves to calculate total lactoferrin within each sample. These concentrations were then adjusted to account for two instances of *in vitro* sample dilution which occurred initially when faecal supernatants were formed and again during serial dilutions.

4.2.4 Faecal egg counts

Quantitative FECs were conducted on all faecal samples used in the ELISA assays. In addition each farm had FECs conducted in the grazing season leading up to sampling with 10 randomly sampled FECs conducted on each of the four to seven sampling visits per farm.

FECs were completed in duplicate, using mini-FLOTAC and fill-FLOTAC devices (University of Naples Federico II, Italy), in accordance with manufacturer's methods. The system works by homogenising the faeces with a floatation solution, allowing it to rest and eggs to float to the top, before skimming off the top layer for microscopic examination. A 1.34 g ml^{-1} flotation solution of zinc sulphate and deionised water (1:1) was created. 45 ml of flotation solution was then mixed with 5.0 g of faeces, in the fill-FLOTAC device. This mixture was then transferred to the two wells of the mini-FLOTAC and rested for ten minutes. The top of the mini-FLOTAC device was then rotated 90°, skimming off any eggs that floated to the top. Eggs were then identified and counted under a microscope. Total counted eggs across both wells of the mini-FLOTAC plate were multiplied by 5x to determine epg.

4.2.5 Dry matter and optical density relationship

Fresh faeces is predominantly made up of water, resulting from the animal's dietary intake. The state of an animal's hydration varies temporally and may significantly influence the water content of its faeces and consequently the concentrations of components, including lactoferrin, within the faeces; essentially *in vivo* dilution of what will later become faecal supernatants.

For the majority of fresh cattle faecal samples, a sub-sample was taken and dry matter content determined by oven drying at 65°C until constant weight. Final lactoferrin results were then multiplied by their dry matter proportion in an attempt to account for the water content of faeces.

Faecal supernatants, all at 1:2 (w/v) ratio of faeces to protease inhibitor, were measured to determine OD, this was conducted twice, on 100 µl and 50 µl of supernatant. Blank 96-well plates were initially read to determine background. Portions of each supernatant were pipetted into individual wells, avoiding the outer two rows and columns, to avoid potential

edge effect. Plates were then read using a plate reader, to determine OD, from which the background value was subtracted. Supernatant ODs were then correlated to lactoferrin concentration and significantly correlated data sets subjected to regression analysis.

4.2.6 Statistical analysis

4.2.6.1 ELISAs

A Grubb's outlier test was conducted to identify any statistically significant outliers which were subsequently removed.

ELISA validity was confirmed through reference material results. For total IgA, IgG, IgM, and lactoferrin, ODs from the 10-point reference material dilution curves were plotted and assays considered valid if sigmoidal curves were produced by the data plots. To assess the validity faecal supernatant as a suitable medium for assays to be conducted on, for each assay, ODs of faecal supernatants must be higher than those of blanks. This was determined through individual 2-sample *t*-tests for each assay. These statistical tests were used to test Hypothesis 1. For all *T. circumcincta* assays (for which no reference material was available), an assay was considered valid if the positive controls were significantly higher than TBST blanks, as determined by a 2-sample *t*-test. This test was used for the acceptance/rejection of Hypothesis 3.

A one-way ANOVA with a post-hoc Tukey test was conducted on total antibody concentrations grouped by antibody isotype and by sample type (faecal or serum). This was used to show how antibody concentrations varied between one another and if they were significantly different between faecal and serum samples. This statistical testing was also conducted on the data set for levels of *T. circumcincta* antibodies. A 2-sample *t*-test was conducted to compare lactoferrin concentrations of faecal and serum samples. These tests were used to test Hypothesis 2.

Pearson's correlations were conducted to identify any associative relationships between the different immunomarkers in the different materials. Pearson's correlations were conducted to assess if antibody and lactoferrin levels correlated with one another. This was conducted

twice, once for the results from faecal samples and again for results from serum samples. To mitigate for type I errors, a Bonferroni correction was applied to each of these correlation sets, reducing the critical *p*-value from 0.05 to 0.018. A second set of 64 correlations were conducted to investigate if any faecal immunomarkers correlated with any serum immunomarkers. A Bonferroni adjustment set the critical value at 0.001 for these tests. Each of the eight immunomarkers in faeces underwent a Pearson's correlation against each of the eight immunomarkers in serum. This was conducted for the 22 instances where paired faecal, and serum samples were available, both taken on the same day from the same individual. These statistical analyses tested Hypotheses 4 and 5.

A one-way ANOVA was used to compare reported lactoferrin concentrations of cattle, sheep, and deer faeces. A post-hoc Tukey test was used to identify between which groups any variation lay. A 2-sample *t*-test was used to calculate if serum lactoferrin concentrations of cattle and sheep were significantly different. These tests were used to provide evidence as to whether there may be immunological differences between the groups and to allude to any possibility of sub-optimal cross-reactivity between bovine lactoferrin antigens and those from other ruminants. This statistical test tested Hypothesis 6.

4.2.6.2 Dry matter and optical density

A Pearson's correlation was conducted to determine the association between sample dry matter and faecal supernatant OD. A second correlation was then conducted to determine if faecal supernatant OD correlated with lactoferrin concentration, this was then followed by a regression analysis of the same factors, with OD as the determining variable. These tests were used to test Hypothesis 7.

4.3 Results

4.3.1 Assay validations

All assays were successfully validated for both their functionality and their ability to work effectively with faecal supernatant. These results led to the acceptance of Hypothesis 1.

4.3.1.1 Antibody assay validations

Ten-point dilution series for total IgA, IgG, and IgM all produced sigmoidal curves (Figure 4.2). These were taken forward and used for the interpolation of ODs from all samples.

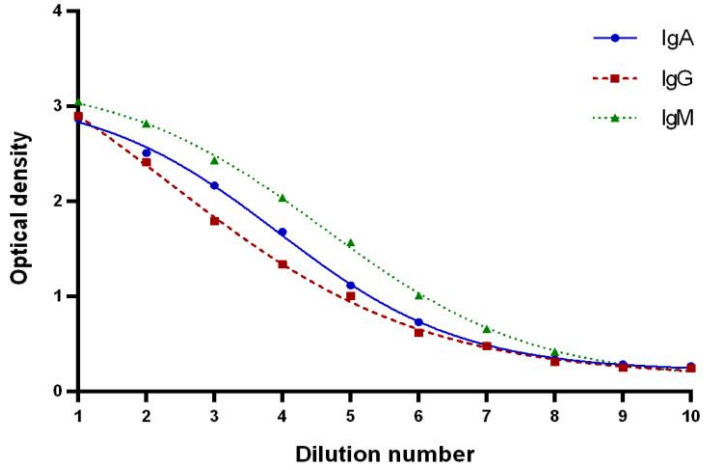


Figure 4.2 - Sigmoidal curves generated from 10-point dilution series of reference material for total IgA, IgG, and IgM assays.

Positive controls for the *T. circumcincta* assays yielded consistent and significantly higher optical densities than the negative controls (Figure 4.3). These differences were confirmed by 2-sample *t*-tests for each *T. circumcincta* antibody, IgA ($t = 25.29$, $p < 0.0005$), IgG ($t = 16.44$, $p < 0.0005$), IgM ($t = 17.79$, $p < 0.0005$), and IgE ($t = 35.39$, $p < 0.0005$).

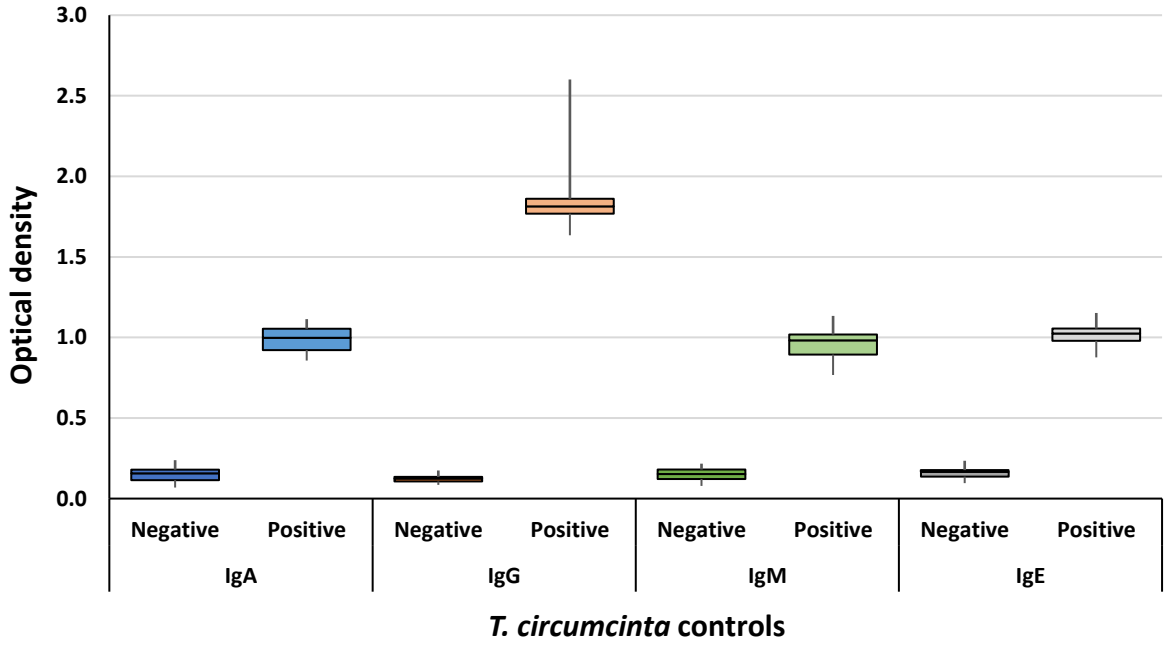


Figure 4.3 - Boxplots comparing negative and positive controls for all T. circumcincta *assays, for purpose of validating the ELISA.*

Faecal supernatant OD values were significantly greater than those of TBST and protease inhibitor negative controls (Figure 4.4; $F = 92.77$, $p < 0.0005$). The exceptions were *T. circumcincta* IgA which was not significantly higher than its protease inhibitor control, and *T. circumcincta* IgE, which was not significantly higher than either of its blanks. Standard errors of faecal supernatatants, and particularly of negative controls, were low. These results mean that Hypothesis 1 was accepted.

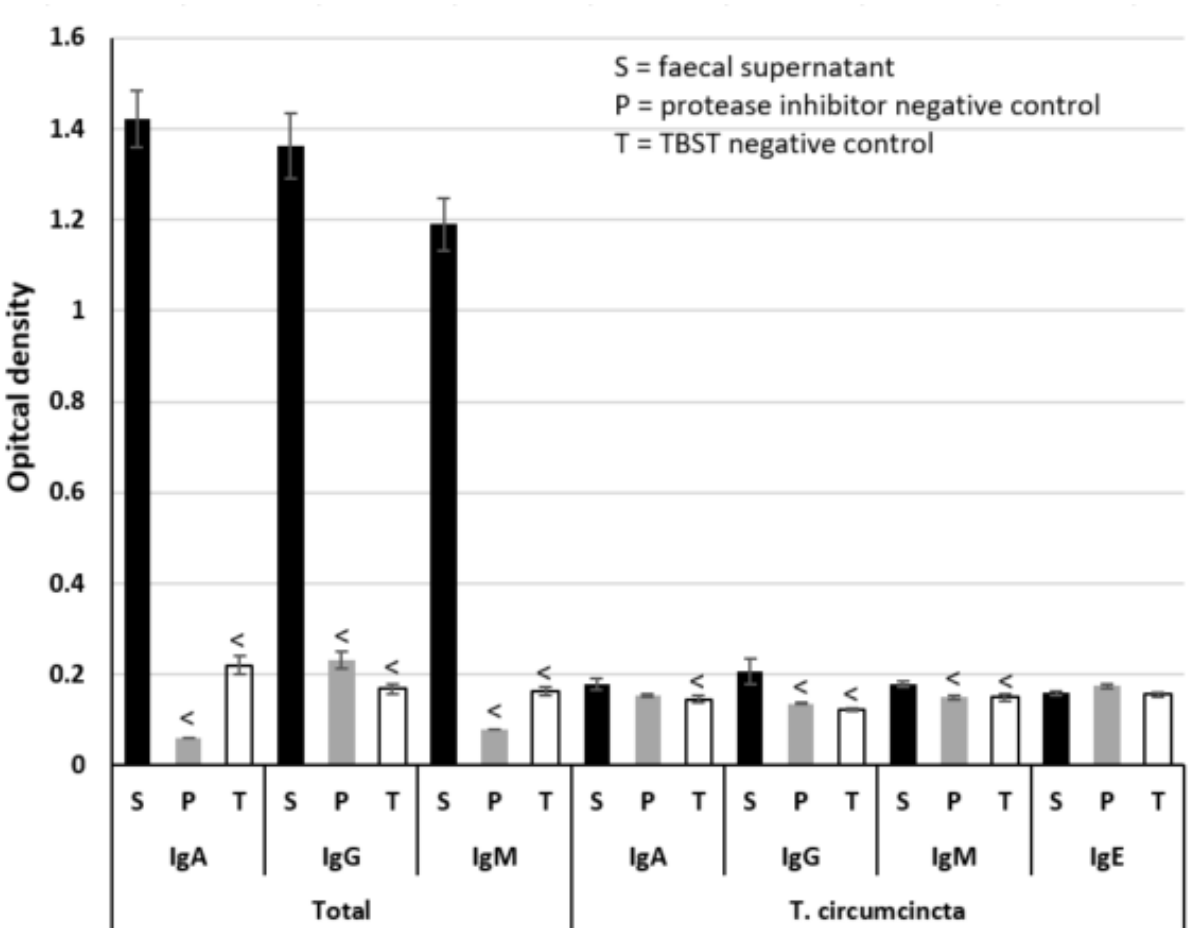


Figure 4.4 - Unadjusted, "raw" optical densities for faecal supernatant, protease inhibitor negative controls, and TBST negative controls, across all assays. Less than symbols (<) above control columns signify that their ODs are statistically significantly less than the faecal supernatant ODs for the same antibody, as determined by a 2-sample t*-test. Error bars represent standard error.*

4.3.1.2 Lactoferrin assay validation

Assays conducted on reference serum produced sigmoidal curves (Figure 4.5). Based on the results of three initial test plates, it was determined that a concentration of 1/2 (50%) was optimum for faecal assays.

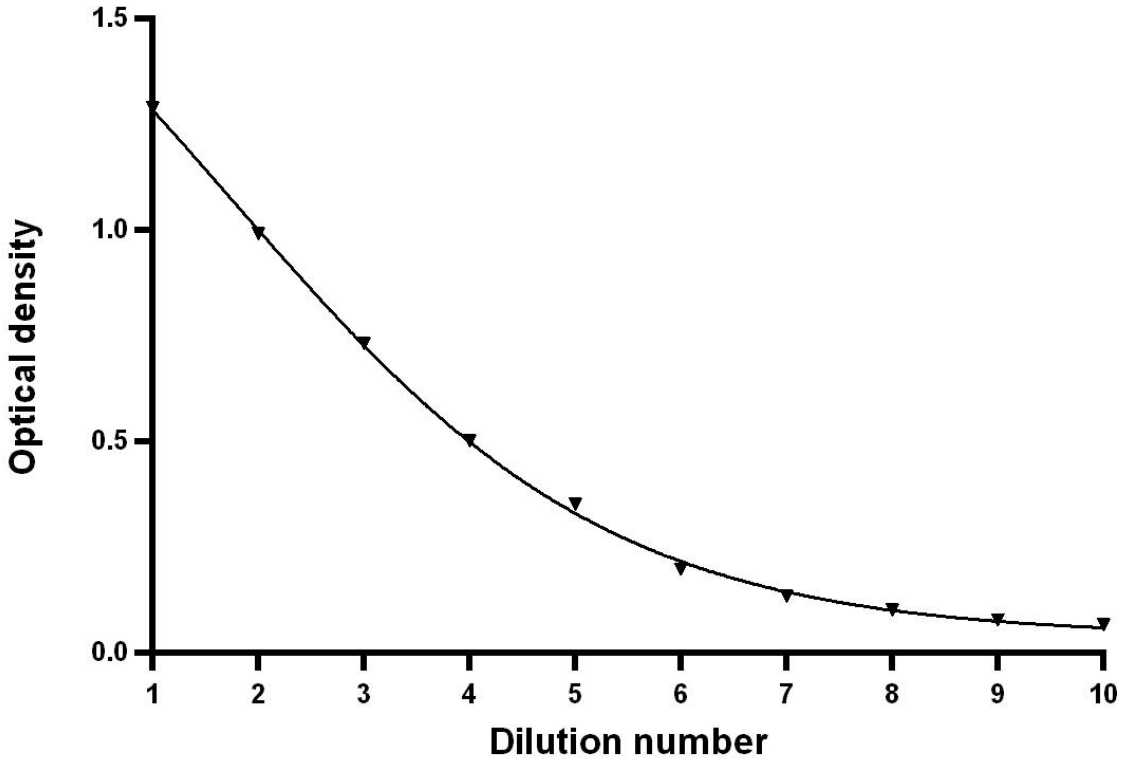


Figure 4.5 - Sigmoidal curve of mean values of assay optical densities, generated from increasing dilutions of reference serum.

A Grubbs' outlier test found one outlier, within the cattle data set, of a recorded lactoferrin concentration 1.94 μg ml^{-1}. This was removed for all statistical analyses and graphing. All faecal samples yielded optical densities significantly above background level as determined of 2-sample *t*-tests comparing background levels to faecal supernatants of cattle ($t = 11.99$, $p < 0.0005$), sheep ($t = 4.72$, $p < 0.0005$), and deer ($t = 8.80$, $p < 0.0005$). These results accept Hypothesis 1.

4.3.2 Concentrations

4.3.2.1 Antibody concentrations

For both total and *T. circumcincta* faecal antibodies, IgA was the most abundant and therefore Hypothesis 2 was accepted.

Total antibody concentrations of all positive samples varied considerably (Figure 4.6). A one-way ANOVA, with a post hoc Tukey test, found that serum antibody concentrations were significantly higher than faecal antibody concentrations (F = 162.21, p < 0.0005). A second one-way ANOVA and Tukey test, comparing just faecal antibody concentrations, found faecal IgA concentrations (mean of 59.7μg ml^{-1}) to be significantly greater than serum IgG and IgM (means of 0.3 and 3.7μl ml^{-1} respectively), which themselves were not significantly different to one another (F = 50.60, p < 0.0005). A third one-way ANOVA and Tukey test, solely comparing serum total antibody concentrations, found that serum IgG concentrations were significantly greater (mean of 10304.4μg ml^{-1}) than serum IgA (mean of 5576.3μg ml^{-1}) and that both were significantly greater than serum IgM (mean of 1684.3μg ml^{-1}) (F = 18.97, p < 0.0005).

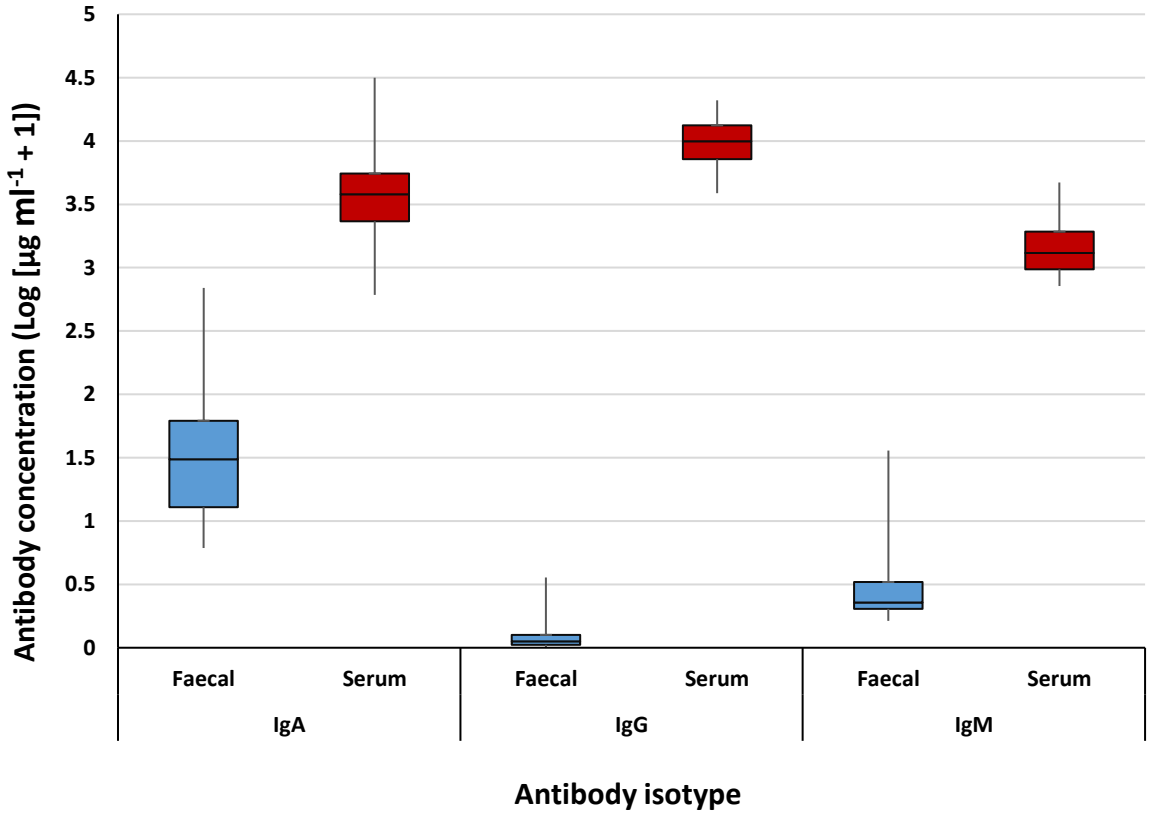


Figure 4.6 - Boxplots of total antibody concentrations (log μg ml^{-1} +1) measured across all cattle faecal and serum samples.

Due to the lack of reference serum and unknown avidities, truly accurate comparisons of *T. circumcincta* antibody assay results were not possible. Nevertheless, comparisons did yield results similar to those for total antibody concentrations (Figure 4.7). As with total antibody levels, when comparing *T. circumcincta* antibody levels in positive faecal samples, *T. circumcincta* IgA levels were significantly greater than *T. circumcincta* IgG, IgM, and IgE (means 0.39, 0.18, 0.20, and 0.18 respectively) (F = 4.00, p = 0.008). Similarly, serum *T. circumcincta* IgG levels were higher than levels of *T. circumcincta* IgA, IgM, and IgE (6075.2, 34.3, 9.18, and 2.9 respectively).

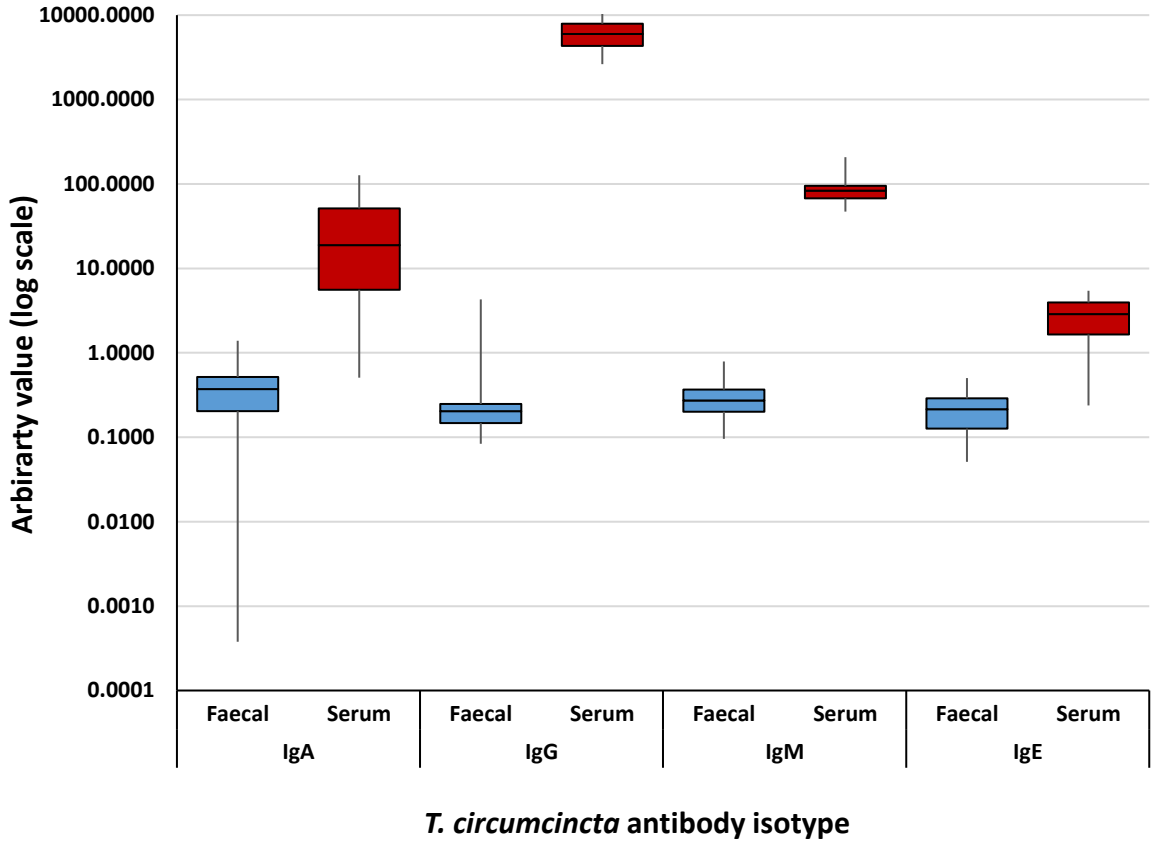


Figure 4.7 - Boxplots of T. circumcincta *specific antibody levels (arbitrary units) measured across all faecal and serum sample. Y-axis is a log scale.*

4.3.2.2 Lactoferrin concentrations

Cattle faecal samples yielded significantly higher concentrations of lactoferrin than sheep and deer (means of 0.26, 0.040, 0.036μg ml^{-1} respectively). A one-way ANOVA found statistically significant differences in faecal lactoferrin concentrations between species (F = 58.25, p < 0.0005) and a post-hoc Tukey test confirmed that cattle samples varied significantly from sheep and deer, which themselves were not significantly different from each other. Such results are visible in the distribution of data for each species (Figure 4.8). As a result, Hypothesis 6 was accepted.

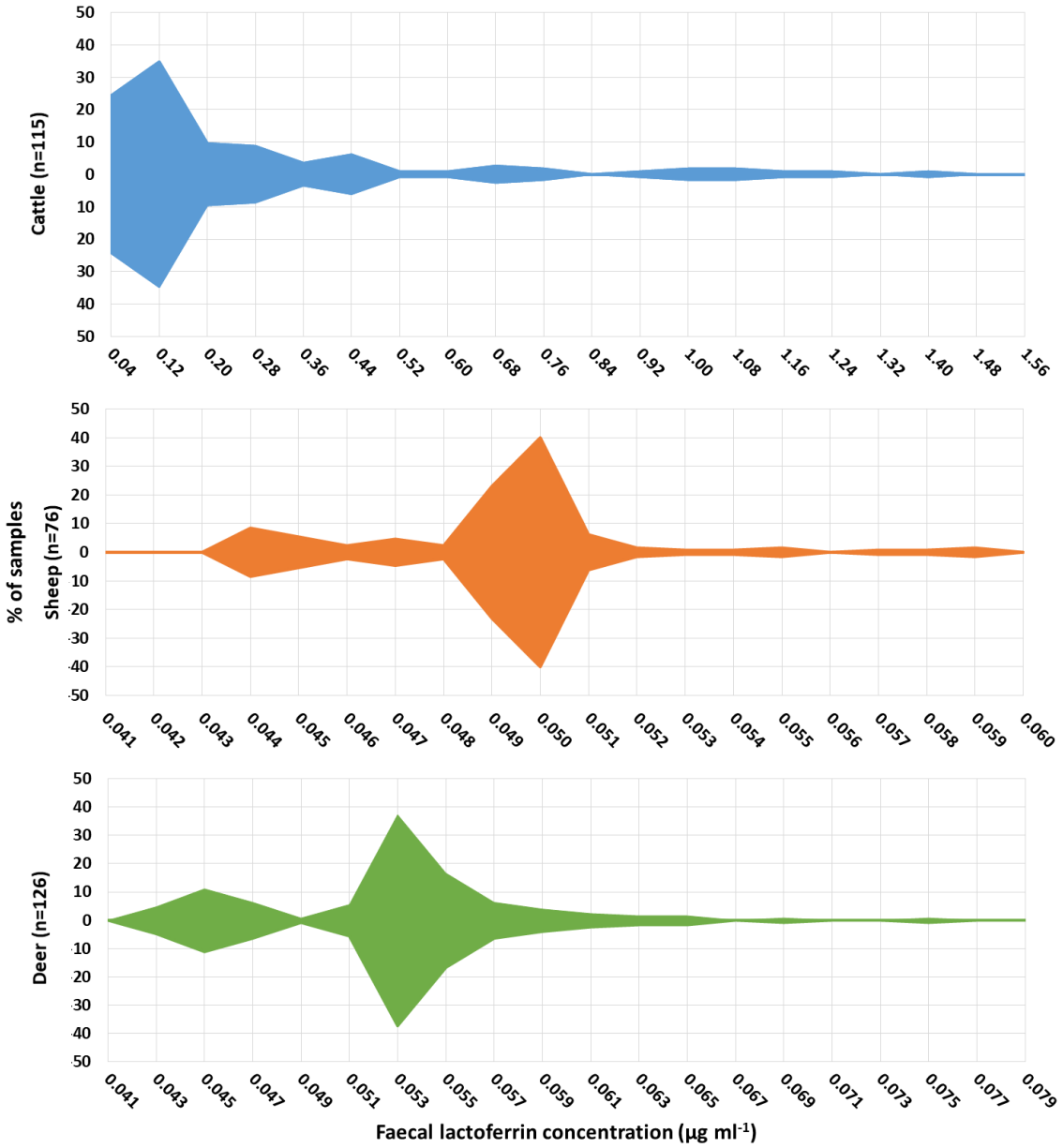


Figure 4.8 - Kite graph showing the distribution of faecal lactoferrin concentrations (μg ml^{-1}) of faecal samples from cattle, sheep, and deer. Note: X-axis is different for each data set.

A Grubbs' outlier test found one outlier, within the sheep serum data set, of a recorded lactoferrin concentration 0.659 μg ml^{-1}. This was removed for all statistical analyses and graphing. As only one deer blood sample was available it was not included in the analysis; the sample had a lactoferrin concentration of 0.097 μg ml^{-1}. A 2-sample *t*-test showed no statistically significant difference between the lactoferrin concentrations derived from cattle or sheep (means: 0.008 and 0.006 μg ml^{-1} respectively) blood serum/plasma (t = 1.64, p = 0.146; Figure 4.9).

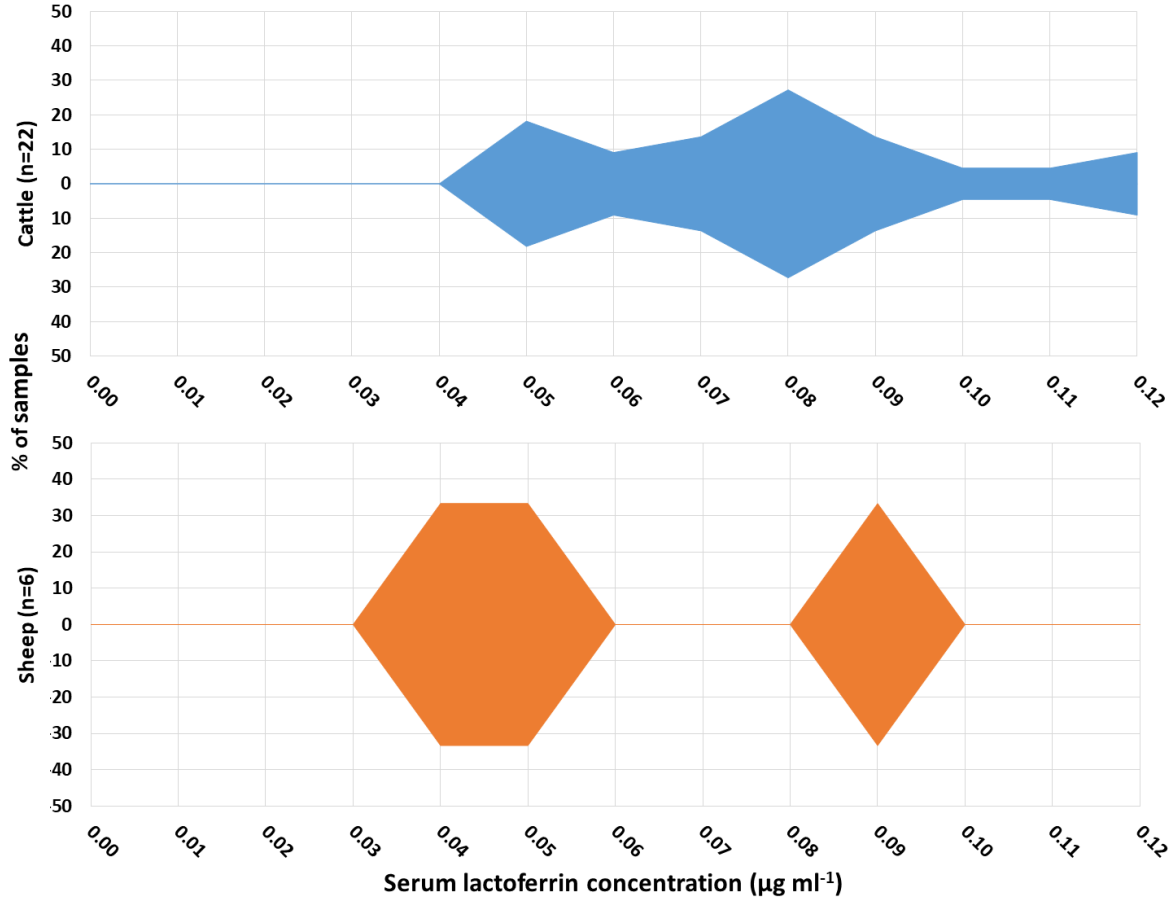


Figure 4.9 - Kite graph showing the distribution of serum and plasma lactoferrin concentrations (μg ml^{-1}) of faeces taken from cattle and sheep.

No statistically significant correlation was found between faecal and serum lactoferrin concentrations taken from the same individuals on the same day (0.069, p = 0.767).

4.3.3 Faecal egg counts

On Farm #1 29.2% of animals had GIN eggs in their faeces, of these animals the mean epg was 17 (s.e. 7.7).

On Farm #2 16.7% of animals were positive, with a mean epg of 139 (s.e. 82.6), however, with the removal of one outlier, with 450 epg, this fell to an average epg of 61 (s.e. 36.1).

Farm #3 had 26.7% of animals recorded as positive, of which the mean epg was 9 (s.e. 1.8).

Whilst differences in faecal egg count levels were observed between sample farms (Figure 4.10), the low number of positive samples made the FEC data inappropriate for further statistical analysis.

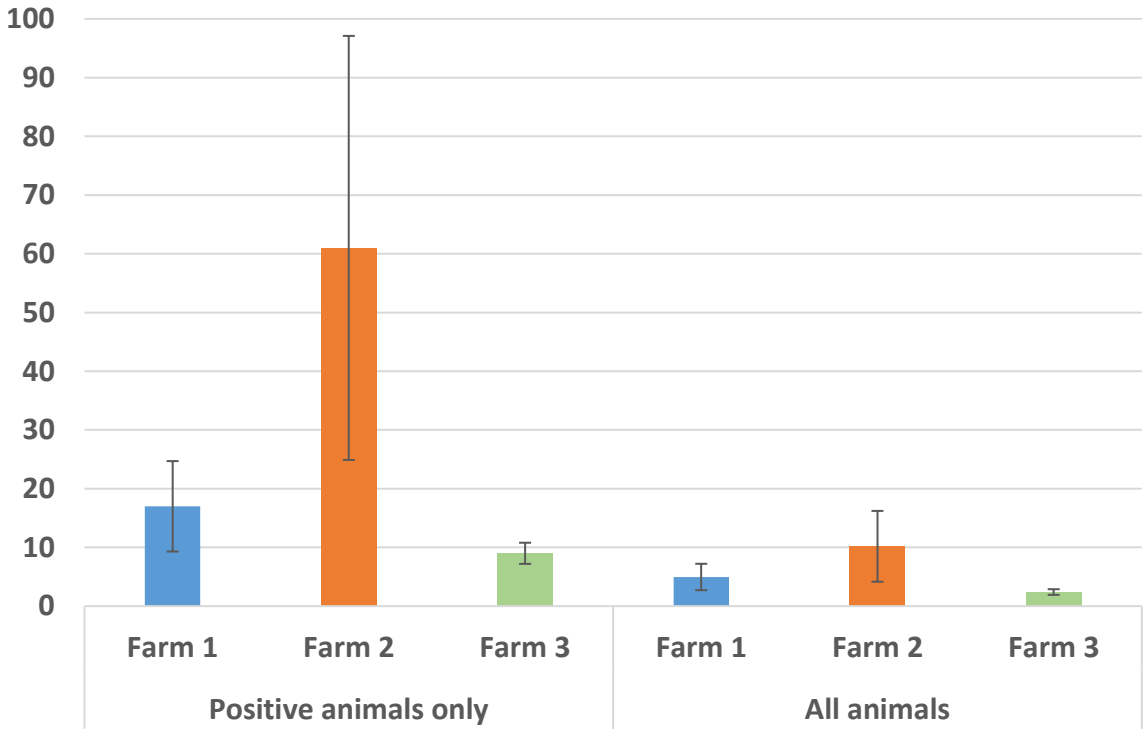


Figure 4.10 - Faecal egg count results from the three farms samples. Results are shown for the positive animals only and extrapolated to also give a whole farm average. Error bars represent standard error.

4.3.4 Correlations

4.3.4.1 Immunomarker correlations

The majority of immunomarker pairings correlated significantly (Table 4.1), however, *T. circumcincta IgE* and lactoferrin were notable exceptions to this. *T. circumcincta* IgE only correlated significantly with *T. circumcincta* IgM and lactoferrin did not correlate with any other immunomarkers analysed.

Table 4.1 – Pearson's correlation results of antibody and lactoferrin levels/concentrations from faecal samples only. "Lfn" = lactoferrin. Top and right side: Pearson's correlation coefficient for each pairing. Bottom and left side: p –value for each pairing. Cells highlighted in green are statistically significant at a p-value, with a Bonferroni correction, of 0.00179.

		Total			***T. circumcincta***				
		IgA	**IgG**	**IgM**	**IgA**	**IgG**	**IgM**	**IgE**	**Lfn**
Total	**IgA**	correl. / p-value	0.562	0.700	0.300	0.651	0.514	-0.050	0.031
	IgG	0.000		0.453	0.161	0.345	0.248	-0.203	0.095
	IgM	0.000	0.000		0.485	0.640	0.530	-0.180	-0.034
T. circumcincta	**IgA**	0.001	0.085	0.000		0.781	0.622	0.079	0.021
	IgG	0.000	0.000	0.000	0.000		0.654	0.011	-0.023
	IgM	0.000	0.007	0.000	0.000	0.000		0.364	0.075
	IgE	0.593	0.028	0.053	0.402	0.905	0.000		-0.064
	Lfn	0.744	0.312	0.715	0.826	0.805	0.429	0.498	

Only one antibody pairing correlated significantly within the serum samples, total IgM vs. *T. circumcincta* IgM. Prior to the Bonferroni adjustment, which reduced the significance level from 0.05 to 0.00179, nine pairings correlated significantly.

Table 4.2 - Pearson's correlation results of antibody and lactoferrin levels/concentrations from serum samples only. "Lfn" = lactoferrin. Top and right side: Pearson's correlation coefficient for each pairing. Bottom and left side: p –value for each pairing. Cells highlighted in green are statistically significant at a p-value, with a Bonferroni correction, of 0.00179.

		Total			***T. circumcincta***				
		IgA	**IgG**	**IgM**	**IgA**	**IgG**	**IgM**	**IgE**	**Lfn**
Total	**IgA**	correl. / p-value	0.487	0.418	0.161	-0.138	0.103	0.359	0.011
	IgG	0.021		0.500	0.530	0.352	0.324	0.332	0.042
	IgM	0.053	0.018		0.439	0.268	0.669	0.445	-0.267
T. circumcincta	**IgA**	0.473	0.011	0.041		0.235	0.370	0.155	-0.276
	IgG	0.541	0.108	0.227	0.291		0.545	-0.110	-0.005
	IgM	0.650	0.141	0.001	0.090	0.009		0.014	-0.452
	IgE	0.101	0.131	0.038	0.490	0.625	0.952		0.053
	Lfn	0.963	0.854	0.229	0.213	0.982	0.035	0.816	

The results of the different correlation analysis, comparing antibody and lactoferrin concentrations, led to the partial acceptance of Hypothesis 4.

4.3.4.2 Faecal and serum correlations

Results of a Pearson's correlations, for the comparison of faecal antibody and lactoferrin levels to those in serum, did not yield any statistically significant results. Consequently, Hypothesis 5 was accepted.

4.3.5 Dry matter and optical density

Dry matter content of faeces correlated negatively with lactoferrin concentration (-0.148) however this was non-significant ($p = 0.161$). Optical density of faecal supernatants correlated significantly with lactoferrin concentration at 100 µl (0.377, $p = 0.004$) but not 50 µl (0.135, $p = 0.135$; Figure 4.11). Subsequent regression analysis of lactoferrin concentration as a response to OD at 100 µl yielded an R^2 of 14.23%. These results support hypothesis 7.

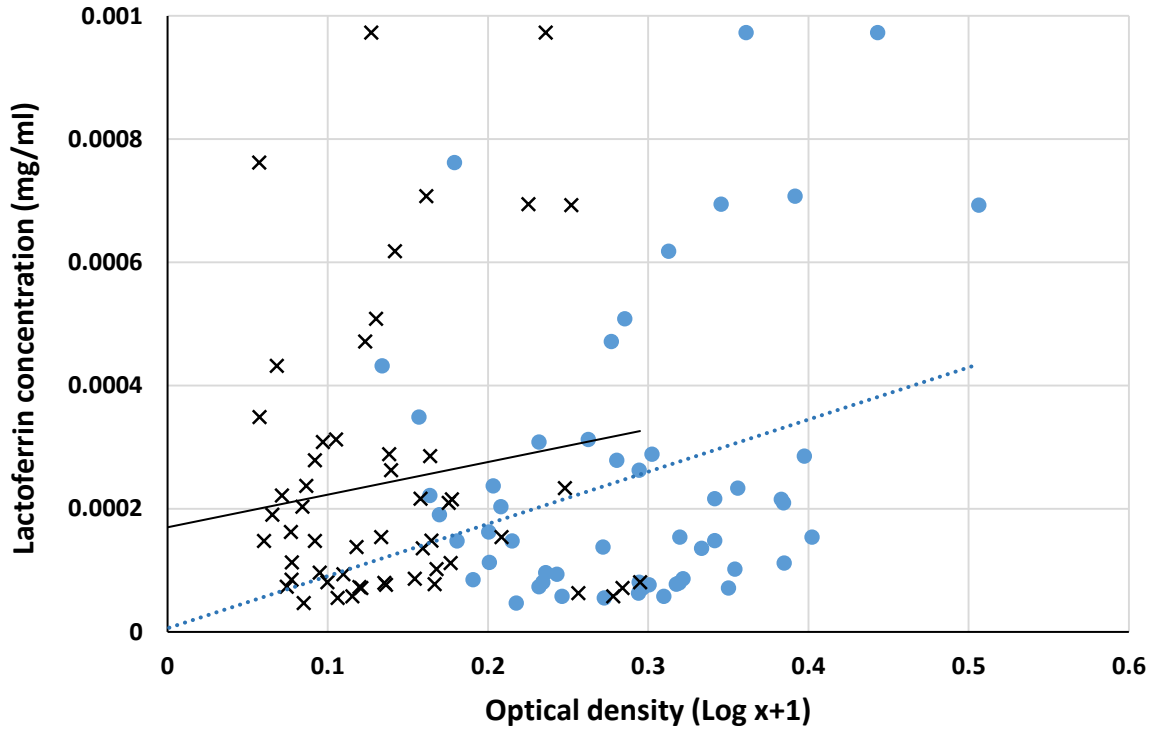


Figure 4.11 - Scatter plot, with an associated trendline, of sample optical density, at 50µl and 100µl volume, and lactoferrin concentration. Black crosses represent 50µl, with a solid black trendline. Blue circles represent 100µl with a dashed blue trendline.

4.4 Discussion

The experiments achieved their primary objective – to detect and quantify antibodies and lactoferrin in faecal samples of cattle, using ELISA protocols. This represents a potential advancement in the field of veterinary diagnostics and creates an opportunity for the development of new diagnostic techniques and products for mainstream adoption in veterinary medicine.

4.4.1 Assay and supernatant validity

The sigmoidal curves produced by reference serum, at varying concentrations, provided evidence that the commercial ELISA products worked effectively and in line with manufacturer descriptions and design. This provided a stable foundation from which to test Hypothesis 1, that antibodies and lactoferrin can be detected and quantified, via ELISA, in bovine faecal supernatant. For all antibody assays, apart from *T. circumcincta* IgE, ODs of faecal supernatants were significantly greater than TBST negative controls. An analogous result was obtained for lactoferrin. These results mean that Hypothesis 1 was accepted and that faecal supernatants, created as per the outlined protocol, are suitable for the detection and quantification of antibodies and lactoferrin, using ELISAs.

The faecal supernatant ODs observed for *T. circumcincta* IgA, IgG, and IgM, were all significantly above control levels, supporting the acceptance of Hypothesis 3, that ovine anti-*T. circumcincta* antibodies can be used for the detection of *T. circumcincta* specific antibodies, by ELISA. This highlights the homology and cross-reactivity of ovine and bovine antibodies, as described by Lewin et al. (1985). Given that *T. circumcincta* is an ovine specific parasite, results supportthe suggestion that anti-*T. circumcincta* antibodies have avidity for other GIN species. This has been observed for *T. circumcincta* antibodies previously (Hayward et al., 2014) and for other species (Molina et al., 1999), however typically for parasites derived from the same host species. The exact avidity of the *T. circumcincta* antibodies to different species remains unknown.

The success of the assay protocols means that results derived from them can also be considered as valid. *T. circumcincta* IgE results for faecal supernatant were not significantly above background, whilst it may simply be the case that the antibody wasn't abundant in samples, it may also highlight a lower detection limit of the protocol. The higher antibody and

lactoferrin concentrations found in serum samples, when compared to faecal samples, supports the latter. Whilst there is still significant advancement necessary to fully understand what results from these methods can show, it is possible to draw certain conclusions.

4.4.2 Concentrations

4.4.2.1 Antibodies

Concentrations of total faecal IgA were 197x that of IgG and 16x that of IgM. Levels of *T. circumcincta* antibodies had a similar but less variable trend with IgA levels being 2.2x that of IgG and IgE and 2.0x that of IgM. These results meant that Hypothesis 2, that IgA is the most predominant antibody in bovine faecal samples, was accepted. This is consistent with the literature, that IgA is the most abundant antibody in mucosal membranes (Hughes et al., 1981; Lamm, 1988; Macpherson et al., 2008). This result supports the hypothesis that bovine faecal antibody levels are indicative of mucosal membrane antibody levels. Whilst, from current research, it is not possible to pinpoint the exact mucosal source of these antibodies, given the formation and passage of faeces, it is proposed that results represent gastrointestinal mucosal membrane status, which would be concurrent with the literature on human faecal antibodies (Baklien and Brandtzaeg, 1975; Bjerke et al., 1986; Crabbé and Heremans, 1968; Tomasi, 1970). A possible mechanism for this is that during gut transit, organic material may accumulate biomarkers from mucosal membranes up until the point it is excreted as faeces. Testing such a hypothesis could be achieved by taking post-mortem tissue samples and washes throughout the digestive tract and subjecting them to similar ELISA analysis and seeing if antibody profiles correlated with those found in faecal material from the same animals. Post-mortem intestinal washes have been utilised in animal model studies to recover antibodies and other biomarkers (Negrão-Corrêa et al., 1996), showing that they can be liberated from mucosal membranes. This work could be further supported by the specific quantification of secretory IgA (sIgA) in faecal material, which would be highly abundant if antibodies were from a mucosal source. The most abundant antibody in serum was IgG, this result is concurrent with the literature (Fahey and McKelvey, 1965; Hughes et al., 1981). The high IgA levels in faeces, compared high IgG levels in serum, was also observed by Watt et al. (2015), providing further reassurance.

4.4.2.2 Lactoferrin

Whilst the research's primary focus was on cattle, the opportunity was taken to complete the lactoferrin assays on samples from sheep and deer. Higher lactoferrin concentrations were found in bovine faecal samples (0.26μg ml^{-1}), compared to sheep (0.040μg ml^{-1}) and deer (0.036 μg ml^{-1}), and meant that Hypothesis 6 was accepted. These differences were potentially driven by host factors such as immunity and disease exposure, however, may be indicative of sub-optimal cross-reactivity of non-bovine lactoferrin to bovine lactoferrin ELISAs. The observation in the similarity in serum lactoferrin concentrations between cattle and sheep, provide support for the argument that the observed differences are, at least in part, truly representative. Whilst bovine lactoferrin is not identical to isotypes from other ruminants, the molecules are structurally similar when contrasted against different interspecies isotypes of other immunological molecules (Shimazaki et al., 1991). The unavailability of lactoferrin reference material for non-bovine ruminants means it is not currently practical to assess the cross-reactivity of non-bovine lactoferrin to a bovine lactoferrin assay. Mass-spectroscopy is an option for addressing this question (Janin-Bussat et al., 2010; Zhang et al., 2014, 2009), however, the initial work to do so would be extremely costly and risky with respect to the cost and time investment it would require. Despite this current uncertainty, the assay can still be used for non-bovine ruminants, so long as results are considered relatively and in the context of the individual species.

The positive skew of lactoferrin concentrations, towards the lower end of their scale, suggests a potential baseline or 'typical' concentration that is being observed. Results outside of this grouping may be considered to have elevated lactoferrin levels, however, if it is not clear as to if those levels are due to innate biological differences or due to disease. As with the antibody results, it is not possible to determine precisely where the detected lactoferrin originated from within the animal, however, this could potentially be achieved through post-mortem study as described above. Given the antibody results, the role of lactoferrin at mucosal membranes, and the use of lactoferrin in human medicine, therefore, a potential source of faecal lactoferrin is gastrointestinal mucosal membranes.

4.4.2.3 Correlations

The majority of immunomarkers correlated to one-another within faecal samples, but not within serum samples. This meant that Hypothesis 4 was not accepted. More importantly, it

suggests the possibility that sample composition may be confounding. Individual differences in faecal composition may, *in vivo*, concentrate or dilute faecal samples. This is somewhat supported by the correlation found between sample optical density of faecal supernatants at 100µl with lactoferrin concentration and subsequent rejection of Hypothesis 7, that faecal supernatant optical density does not correlate with lactoferrin concentrations. However, serum samples also yielded a number of significant correlations, supporting the use of faecal supernatant. Immune responses do not happen in isolation and it is therefore not unexpected to for antibody levels to rise and fall with one another.

Hypothesis 5 was that antibody and lactoferrin levels in faecal samples do not correlate to those in serum samples. This hypothesis was accepted because of the general lack of correlation between immunomarker levels in blood and faeces show that the method is not a replacement or proxy for measurements of systemic antibody levels. Instead, results support the utility of the assay to derive specific information about animal health that cannot easily be obtained otherwise. This information may prove to be of greater use and relevance for the assessment of GIN derived, and other, gut damage. As a result faecal immunomarker, ELISA methods could best be implemented as part of comprehensive health assessments for the purpose of informing TST strategies and selective breeding in situations where GIN and other gastrointestinal infections are a primary driver of system loses.

Only 15% of faecal samples were returned as positive after FEC, providing an inadequate amount of positive data to determine with any certainty, if a correlation exists between nematode egg counts and faecal antibody levels. The negative correlations observed (although non-significant) are concurrent with observations by Watt et al. (2015). The lower faecal antibody levels and lack of correlation with FECs may stem from hypobiosis as samples were taken during late autumn and early winter (Capitini et al., 1990). A longitudinal study, tracking seasonal faecal antibody levels would clarify this.

4.4.3 Benefits

The outlined methods are entirely non-invasive, providing an immediate welfare benefit to the animal, but also provide practical and financial benefits. It is not necessary to arrange and pay for a veterinarian to take blood samples, saving money and allowing for greater flexibility. There is therefore also no need to put the animals through a run or crush, reducing stress and, therefore, welfare. These benefits also facilitate repeat sampling. In a research context, invasive procedures in the UK require Home Office licensing, which can be a lengthy process

and may place limitations on sample numbers and other aspects of experimental design. The outlined methodologies do not require such licensing and therefore can be conducted more liberally and by unqualified individuals such as students and farm staff. Benefits extend beyond their use in agriculture, the study and health assessment of animals can be difficult for species which are evasive, elusive, or dangerous. In these situations, drawing blood samples may not be practical or safe and, therefore, the ability to utilise faecal diagnostic tools would be of great value.

4.4.4 Future development potential

Molecular diagnostics tools, such as ELISAs and similar technologies, have the potential to be significantly advanced for use in veterinary medicine, as has been achieved in human medicine. The technology to accomplish this already exists and is routinely utilised in other areas of human and veterinary medicine. With adequate resources and demand, this could be realistically achieved and rolled out.

Similar recent advances have seen the development and adoption of salivary antibody tests, for the study of GINs in sheep (Shaw et al., 2012). Carla® antibodies bind to Carla® molecules found on the surface of all internal parasite larvae of livestock. The Carla® Saliva Test detects Carla® antibodies (Harrison et al., 2003) in sheep saliva, however, these antibodies are also present in gastrointestinal mucus, meaning that faecal antibody detection may be a suitable approach for measuring Carla® antibodies. The primary disadvantage of a salivary test is the necessity to restrain and perform an invasive procedure on the animal, however, it highlights a progressive adaptation of known techniques and the principles could be applied to faecal material. Research and development of the outlined methodologies and associated technologies, using advancements on salivary antibody tests as a template, has the potential to create a highly practical and informative diagnostic method.

One of the most common diagnostic methods for the parasitic disease schistosomiasis is a circulating cathodic antigen test (CCA), which is based on the principles of ELISA methods. A CCA is a simple device, similar to a pregnancy test in both design and function. Antigens in the urine of patients are administered to the device, as the urine is absorbed the antigens move through the device and are captured on strips of complementary capture antigens, which produce colour upon reaction, indicating a positive sample. The success of faecal

immunomarker detection in this research highlights the possibility of the development of rapid molecular diagnostic tools, such as a CCA, for mainstream adoption in veterinary medicine. Such tests could be used for rapid and cheap on-farm diagnostics, which could easily be implemented by farm staff. The main scientific hurdle to this is the identification and isolation of parasite antigens, the results of which would influence how specific such a test could be in identification or GIN species. Once that is achieved the antigens could be produced as part of a CCA-style device and used with a faecal dilatant in the place of urine, in order to pick up markers of GINs. As with schistosomiasis, the method could be more effective and rapid than FEC techniques (Sousa-Figueiredo et al., 2013), whilst simultaneously requiring fewer resources and expertise.

4.5 Conclusion

This work complements and furthers that of Watt et al. (2015) through the adaptation and application of faecal immune marker protocols to cover cattle. Consistent positive results, above background levels, combined with the range and distribution of results, support the methodology as a valid immunological tool. Results indicated that faecal antibody levels are representative of gastrointestinal immunology and therefore the outlined methodology has the potential to provide novel and unique information about gastrointestinal health and immunology of cattle and other ruminants.

This advancement represents a promising new tool to assess immunological aspects of ruminant gut health in a timely and cost-effective manner. The method is highly ethical as it is non-invasive, which harbours the additional benefit of not requiring trained veterinarians or licensing. In addition, the method has a high throughput and is applicable to all individuals. For more comprehensive interpretation of faecal antibody levels, further work needs to be performed to determine the drivers of faecal antibody concentrations, most notably the role of pathogens. The successful protocols within this study and by Watt et al. (2015), suggest that the methods would be more widely applicable to other mammals, particularly ruminants. Further advancements in the detection of faecal immuno-markers could, in the future, become part of a comprehensive tool-kit for the assessment of animal health and development of disease prevention strategies.

Chapter 5

Mob mentality – A case study assessing system metrics of mob-grazing farms

Summary

Mob-grazing is a method of rotational grazing management which is characterised by rapidly moving cattle through small grazing 'cells' at a high stocking density. There are numerous reported benefits of mob-grazing and, whilst they are founded on scientific principles, are mostly anecdotal. Such benefits include increased pasture productivity, improved soil quality and herd health. Despite its increasing implementation, there is very little scientific information about mob-grazing or other similar forms of rapid rotational grazing. A season-long case-study was conducted investigating three UK mob-grazing farms in South West England and Scotland to assess metrics of soil quality, forage quality, pasture performance, and parasite burdens under different climate scenarios. All results were either neutral or in favour of mob-grazing when compared to a control farm. Notably, there was a trend towards improved pasture performance and lower parasite burdens on the mob-grazing farms. Results indicate that mob-grazing holds the potential to be a useful grazing strategy to effectively utilise the underlying biological potential of grazing livestock systems. Specific system aspects are discussed and the necessity for further specific research into these are outlined.

5.1 Introduction

Chapters 2 and 3 have explored the impact of various management decisions on the environment and local ecosystem, achieved through a combination of computer modelling and controlled field studies. This research has highlighted the system-level impact that seemingly minor management decisions can have on a wide range of factors such as soil quality, biodiversity, and animal health. The control of the use of anthelmintics, along with the benefits it can yield, have been outlined and supported through Chapters 1-4. However, an effective parasite management strategy must not solely focus on veterinary intervention, as is far too often the case, but must also consider 'natural' or integrated control methods. This can include a range of factors, such as managing cattle in relation to epidemiological (often climate-based) forecasts, ensuring hygienic housing, and the movement of cattle on farm in relation to parasite life-cycles. Whilst the approaches taken in Chapters 1-4 hold merit, to further understand system-level impacts of management it is necessary to scale research up one more level and generate case study evidence from independent farms practicing relatively novel and unconventional management.

Mob-grazing (also known as "pulse grazing" and "strip grazing") is a method of managing pasture-fed livestock through rotational grazing. The oldest literary mentions of mob-grazing were by Levy (1950, 1949) who, having talked to over 10,000 farmers throughout the Commonwealth, suggested that mob-grazing was the best practice for 'hill country' farming (the farming of deforested hillsides). The passing nature of the mention, with limited description, suggests that the method was common knowledge at this point, particularly in Levy's home country of New Zealand.

The technique has been gradually increasing in popularity over the last few years – particularly within the organic sector. Mob-grazing is characterised by the sub-division of grazing fields into smaller units, known as 'cells', through which livestock graze for short periods before being moved to the next cell (Figure 5.1). The result is that cells are grazed intensively for very short periods before having a prolonged fallow period. This is somewhat analogous to how wild bovines naturally graze and roam, moving continuously on a route, never returning back to the same pasture in that season. One of the core principles of mob-grazing is that the increased rest periods promote herbage growth when compared to

conventional grazing systems, allowing plants to complete more of their life-cycle and 'harvest' more sunlight due to an increased leaf area for photosynthesis. This supposedly mitigates the lack of external nutrient inputs and also yields a number of indirect benefits.

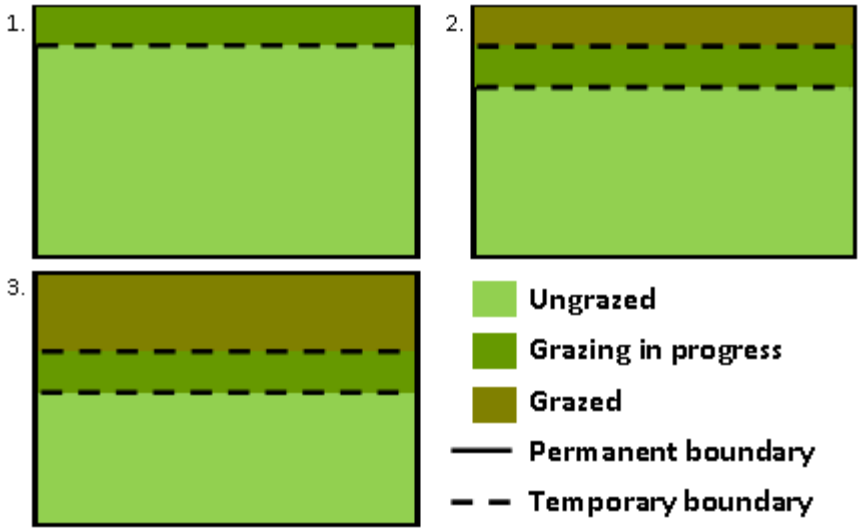


Figure 5.1 - Diagram of three sequential stages of a mob-grazing rotation. Field 1 represents a field undergoing grazing of its first cell. Field 2 represents a field undergoing grazing in its second cell, with the first having been grazed. Field 2 represents a field where the third cell is being grazed. Source: original.

Whilst there has been a lot of academic research into rotational grazing in general (Barger et al., 1994; Barrett et al., 2001; Briske et al., 2008, 2011; Eysker et al., 1993; Marley et al., 2007; Stobbs, 1969; Walton et al., 1981), there is a distinct lack of research specifically into mob-grazing or variants thereof. This is significant for a number of reasons, firstly, mob-grazing is becoming increasingly popular and there is, therefore, a need to understand the economic and environmental consequences of this. Secondly, mob-grazing is one of, if not the, most extreme example of rotational grazing in terms of the fast rate at which cattle are moved between fields. Given the debates surrounding rotational grazing, investigating this extreme has the potential to draw impacts and generate a more rounded and comprehensive understanding of rotational grazing systems. A range of farmer orientated newspapers, magazines, and online articles outline the supposed benefits of mob-grazing. Whilst these are by no means academic pieces, they are a valuable resource – agricultural research is most effective when considered in respect to the end user and thus views and trends within the farming community are equally important as scientific evidence.

Although currently in a state of growth (Soil Association, 2017), the organic sector is particularly turbulent and highly subject to external drivers, such as economic turmoil. From 2007 to 2009, the height of the world economic crisis, UK land use for organic farming fell by nearly 25% (DEFRA, 2017), as farmers wanted to secure their income sources in fear that consumers would no longer be willing to pay the premium that organic produce demands. This highlights some of the insecurities that can be associated with organic farming and, therefore, any way in which these systems can be more secure would improve a farm's long term sustainability and resilience. As a management technique, mob-grazing may harbour the ability to address these concerns and provide farms with added security with regards to their economic sustainability through economic cycles.

5.1.1 Potential advantages

There are hypothetical potentials for mob-grazing to yield a variety of benefits within grazing livestock systems, however, this has not been investigated in a scientific context, and therefore the reported benefits of mob-grazing are mostly anecdotal. Some of these anecdotal benefits are described and explained below, however, they have not been scientifically studied in the context of mob-grazing. Core to the ethos of mob-grazing is the enhancement and utilisation of natural biological processes within the farming system and the associated benefits of enhancing biological activity.

5.1.1.1 Parasite epidemiology

Farmers who use mob-grazing often state that their animals are healthier, citing a reduction in veterinary intervention. One claim is that cattle are consuming healthier and more nutritious parts of the plants, not being forced to consume indigestible stems (Chapman, 2012). A farmer from Colorado claimed that mob-grazing had led to his cattle grazing healthier plants, nearly eliminating the need for mineral supplementation. Furthermore, the burden of parasitic horn fly (*Haematobia irritans*) has reduced as the eggs deposited in cattle faeces take two weeks to hatch, at which point cattle are "*a mile away*" and, without a blood meal, the flies die (Thomas, 2012). This example highlights how pathogen life-cycles can be 'broken', as described in Chapter 1 (1.2.1.2), which is particular relevant to the control of GINs and other helminth parasites. Gastrointestinal nematode diseases of cattle are a major cause of losses and negatively impact animal health, however, there are reports of mob-grazing

mitigating against parasitic diseases (Youngs, 2012). Indeed, there is a scientific basis supporting the potential for mob-grazing to mitigate that impact. A typical GIN life-cycle requires eggs to be deposited in faeces, onto pasture. After deposition eggs take a number of weeks to hatch and moult through larval stages to become infective – this is the point of peak pasture infectivity. Conventional grazing systems typically require cattle to graze on the same pasture for weeks at a time, meaning that cattle are exposed to pasture with the highest risk of infectivity. As mob-grazing systems typically move cattle at least once every few days and leave months between returning cattle to the same pasture, cattle miss the point of peak infectivity. Therefore, the life-cycle is partially 'broken', reducing overall incidence and associated losses (Stromberg and Averbeck, 1999). Whilst trematode and cestode lifecycles are more complex, they still require similar extended periods for maturation, therefore, the benefits could extend beyond the control of GIN parasites, but potentially other species of helminths. A number of experiments investigating rotational grazing as a method of parasite control have found supporting evidence. Larsson et al. (2006) found that rotational grazing of cattle can be almost as effective as regular anthelmintic treatment for parasite control. This is supported by Marley et al. (2007), who found that rotationally grazed lambs had lower parasite burdens and higher live weights than conventionally reared lambs, and also by Barger et al. (1994), who found that rotationally grazed goats had FEC results of less than half that of set-stocked goats. However, other experiments have found no significant difference in parasite burdens between set-stocked and rotationally grazed animals (Eysker et al., 1993; Kunkel and Murphy, 1988).

5.1.1.2 Pasture productivity

The primary reported benefit of mob-grazing is an increase in pasture productivity as a result of prolonged rest periods and shorter grazing periods. A short grazing period ensures that herbage is not grazed down to a low level as conventional systems (cattle typically graze to a height of approximately 4-5cm above ground level (Grant et al., 1996; Hoz and Wilman, 1981) and, therefore, leaving significant above ground biomass. The consequence of this is that the proportional re-growth required by herbage is diminished in respect to the plant's total biomass and photosynthetic area. The Angus Beef Bulletin and associated Angus Journal reported on a rancher in Missouri (USA) who made a switch to mob-grazing and subsequently reported substantial benefits. Stocking rate increased as cattle were moved twice per day on

a 140-180 day rotation. The prior system of management-intensive grazing required a $5000 annual spend on clover seed and used considerable quantities of hay, both of which were eliminated entirely after the shift to mob-grazing. Land productivity increased from 0.22 animal units per hectare to 0.45, whilst labour requirements decreased (Kidwell, 2010). Similarly, a farmer in Canada has claimed that changing to mob-grazing has increased his stocking density four-fold (Chapman, 2012). The Angus Beef Bulletin also interviewed an "educator in holistic management" in South Africa who described benefits of mob-grazing to soil and flora. He claimed that the trampling of herbage protects the soil from erosion and sun damage, helping to maintain a more consistent soil environment. He also suggests that as mob-grazing typically removes less of the plant material in one go, the plant's ability to regrow is increased due to a high remaining surface area for photosynthesis (Thomas, 2013a, 2013b). Whilst these claims may sound extreme and are not necessarily supported by direct scientific evidence, they do highlight mob-grazing's potential and deserve further investigation.

There is scientific evidence supporting the reasoning that increased plant mass and leaf area lead to increases in gross plant growth, however, the relationship is not necessarily linear with relative growth rate decreasing over time (Koyama et al., 2009; Weraduwage et al., 2015). However, a decrease in relative growth rate does not necessarily mean a decrease in total productivity. According to a meta-analysis of rangeland rotational grazing by Briske et al. (2008), 87% of studies reported that rotational systems produced more herbage than conventional systems and 92% found higher levels of animal production.

There are also a variety of potential secondary benefits of improving pasture productivity as a result of increasing herbage productivity. A high cover of herbage can trap moisture, create shade, and act as a habitat for a host of organisms. Moisture is positively associated with soil microbial activity (Barros et al., 1995; Cook and Orchard, 2008) which, in turn, is positively associated with soil health (Arias et al., 2010). Grazing livestock can have a significant impact upon invertebrate species that live on and just above the soil surface, by a negative influence on habitat space, shelter, and temperatures (Hutchinson and King, 1980). Maintaining a high herbage cover can mitigate these effects and enhance invertebrate populations. Such invertebrates play vital roles in the turnover of organic matter and nutrients deposited in dung, incorporating them into the soil where they become readily available for a range of

biological process (Edwards et al., 1973; Lavelle et al., 2006), including herbage production. Increased biodiversity of flora and fauna is something that is widely reported across mob-grazing farms (Gordon, 2010; Kidwell, 2010). However, Briske et al. (2011) found that rotational grazing strategies may not always be ecologically effective, highlighting the benefits of informed and contextual management.

Larger plants require more complex and substantial root systems, this leads to an increase in soil organic matter (SOM), which itself enhances soil biological activity (Hamilton and Frank, 2001; Helal and Sauerbeck, 1986; Rasse et al., 2005); widely regarded as a direct indicator of soil quality. Enhanced root structures also improve soil structure (Kell, 2011), providing resilience in the event of flooding events and alike. Improved pasture productivity and soil quality can reduce the dependency on external inputs such as fertilisers and bought-in silage (Maeder et al., 2002). Furthermore, this can yield the potential to prolong grazing seasons by ensuring more herbage biomass is available leading into periods of slower growth, allowing for larger herds and increased system resilience to adverse events (Helgadóttir et al., 2016). Combined, this could improve the environmental and economic sustainability of mob-grazing farms. A subsequent effect of the aforementioned benefits is an enhancement of local ecology, particularly system biomass and biodiversity (Bengtsson et al., 2005; Stockdale et al., 2001). Such benefits are commonly observed across organic farming systems, however, mob-grazing may be able to further enhance these additional benefits.

5.1.1.3 Dung distribution

Another key impact of mob-grazing and similar intensive rotational strategies is on the distribution of dung and urine. A herd rapidly moving through grazing cells will spatially distribute excreta very evenly, meaning that each area of the farm will receive similar amounts of excreted nutrients as there will not be the 'hot-spots' that are characteristic of less intensive systems. This was found by White et al. (2001) who observed that, within intensive systems, dung was evenly distributed on pasture and that this necessitated less expenditure on manure management. Whilst the exact impact of this is unknown, it is likely to be significant, given the importance of dung in the recycling of nutrients and pasture productivity. Williams and Haynes (1995) found that soil nitrate, phosphate, and organic carbon levels were higher where dung was deposited, even a year later. Similarly, Aarons et al. (2009) found that dung deposition increased soil levels of phosphorous, nitrogen, and

microbial biomass carbon in the immediate area around and under the dung. The even distribution of these impacts across a farm would likely produce a net benefit by improving the utilisation of these nutrients and reduced losses into the wider environment.

5.1.2 Potential disadvantages

The reported disadvantages to mob-grazing are predominantly based around practicality and have received little attention anecdotally or scientifically. One of the major current disadvantages of the technique is the lack of scientific evidence available, meaning that there is a significant risk involved if farms wish to change their management technique to mob-grazing.

5.1.2.1 Labour and resources

The regularity of livestock movement can require significant hands-on management. The predominant downside of this is the time it takes, however, it also reduces the flexibility that a farmer may have with their time. This issue is can be partially addresses by the use of 'smart' fencing systems. Automated gates are becoming more common, these are typically spring loaded gates which are manually closed but unlatch on a timer, and as a result they do not require electricity. More sophisticated gates are available that can open and close through remote control, but they are more costly and require a power source. Another system than could be utilised are 'virtual fences' which require no physical barriers, but instead each animal is fitted with a GPS-enabled collar which provides a negative stimulus to the animal if it begins to stray outside of an area digitally mapped by the farmer.

A second infrastructure related issue is the need for cattle to be able to access water. The small size of cells mean that the total number of grazing areas on mob-grazing farms is high. Therefore, having a water supply in each cell is not practical. One solution for this is by installing ram pumps, which do not have motors and utilise flowing water or gravity to provide energy for the distribution of water. It is also possible to manage the locations of cells and troughs so that one trough can be utilised by numerous cells or to use a portable trough system mounted on a trailer (Figure 5.2).

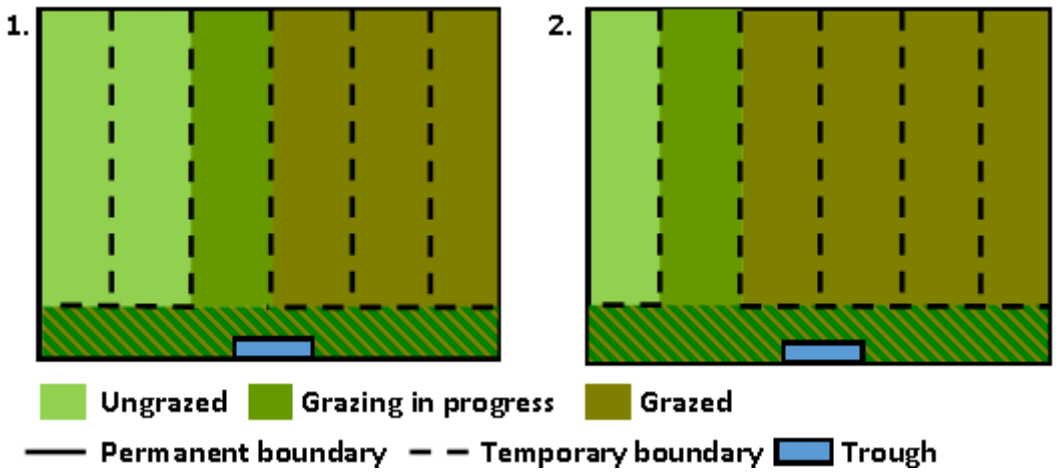


Figure 5.2 - Organisation of grazing cells and troughs so that multiple cells have access to one trough. Field 1 and 2 represent the same field on consecutive livestock movements.

5.1.2.2 Cattle health and welfare

Whilst the rotational nature of mob-grazing may hold benefits for parasite control, the distribution of dung within sub-systems could have a negative impact on disease in general. The high stocking densities mean that dung is distributed relatively evenly across a small area with a large number of cattle. This means that cattle are more likely to come into contact with dung and that their movement may be impeded by the proximity of other cattle. Both of these factors may reduce the ability for animals to practice their natural grazing selection behaviour, which often includes the avoidance of forage adjacent to dung patches (Bao et al., 1998; Forbes and Hodgson, 1985). In addition to helminths, a range of other livestock pathogens can be transmitted through dung, most notably *Mycobacterium bovis*, the causative bacteria of tuberculosis (TB) (Duffield and Young, 1985; Phillips et al., 2003), which is a devastating disease to UK and worldwide livestock production. However, a primary mechanism for reducing the TB infection potential of cattle dung is through the degradation of dung by invertebrates (Phillips et al., 2003). Chapters 1-3 outline evidence surrounding the impact of livestock management, such as the use of anthelmintics and pasture management, on invertebrate activity and dung degradation. Consequently, an organic, holistic, mob-grazing system could produce dung that degrades quicker than conventional systems, therefore, shortening the time that *M. bovis* and other pathogen contaminated dung is of risk to cattle.

The relatively high stocking densities of mob-grazing cells may act as a stressor to individuals by limiting their behaviour. Within herds, certain individuals will exert social dominance over others through using physical force to control space, instilling fear and stress (Beilharz and Zeeb, 1982; Friend and Polan, 1978; Schein and Fohrman, 1955; Wierenga, 1990). The impact of this on the sub-ordinate individual is two-fold. The impact of stress has the potential to reduce individual health and performance, whilst they may also have restricted access to better resources (forage, water, etc.) due to the dominance of others (Grant and Albright, 2001; Phillips and Rind, 2002). However, the majority of this research has been conducted on housed dairy cattle and, therefore, is not necessarily applicable to mob-grazing or other grazing systems.

5.1.3 Research objectives

This research aims to take a broad look at mob-grazing within the UK through case-study research of practicing mob-grazing farms. The objective is to generate a baseline assessment of these farms with respected to the primary reported and potential benefits of intensive rotational grazing. Results will go towards determining if mob-grazing systems may have a practical potential for wider use and if there is a necessity for further research into such systems.

Hypothesis 1 – Cattle from mob-grazing farms have a lower parasite burden than those from a conventional control farm.

Hypothesis 2 – Fields of mob-grazing farms have a greater mass of dry matter herbage (per area) than a control conventional farm.

Hypothesis 3 – The nutritional composition of forage from mob-grazing farms will differ from that of the control farm.

Hypothesis 4 – Soils from mob-grazing farms have more organic matter than soils from a control conventional farm.

5.2 Methods

5.2.1 Case study farms

Three mob-grazing farms were chosen to be part of the case study, along with one conventionally managemed farm. Each farm was visited multiple times for the collection of forage, soil, and dung samples.

5.2.1.1 Farm A

Farm A was an organic beef farm (Soil Association certified) in Cornwall, UK. The farm had two herds of beef cattle. The herd which was observed comprised of 36 cows and 18 calves, predominantly Aberdeen Angus crosses, with three Belgian Blue/Holstein crosses and three Herefords. The other herd grazed the same land, but were never adjacent to the study herd, and comprised of 32 months old, and 12 months old, Aberdeen Angus. Pasture is a species-rich ley comprising of chicory, rushes, sweet vernal, Yorkshire fog, crested dog's-tail, marsh foxtail, dock, thistles, various wildflowers, and more. The ley had been in place for more than five years. The rotational system comprised movement of cattle once daily through half acre cells on a 70-day cycle. This was a refinement on the previous year in which a 120 day cycle was practiced. Cattle were typically on pasture from March until mid-November and housed the rest of the time. Calving primarily occurs in spring, with some later calving in June. Calves are weaned at nine months, which typically falls over December or January.

The farm practices minimal intervention. The only supplement is rock salt which is readily available to the cattle. There is no medicinal prophylaxis or vaccination in use and medicines are only used when infirmary is observed (anecdotally or formally). However, medicinally induced ovulation is practiced. Since beginning mob-grazing, the only notable disease incidence was the death of one individual from an unidentified viral infection. Sampling took place on 16/06/16, 14/07/16, 22/08/16, 12/09/16, and 07/10/16.

5.2.1.2 Farm B

This farm is in Hertfordshire, UK, and is an organic beef farm (Soil Association certified). The herd is comprised of approximately 220 cattle, 120 of which were calves. The overwhelming majority of the cattle were of the Sussex breed. The sward is predominantly perennial rye-grass, however, has timothy-grass, meadow foxtail, bentgrass, and other species, mixed in. This lay has been present for more than five years. Cattle are typically moved daily, however,

this rotation was slowed to approximately one week when bulls were introduced into the herd for mating towards the end of summer. It typically takes around 60 days for cattle to return to pasture which they had previously grazed.

The farm practices minimal intervention, in line with Soil Association guidelines and uses no fertilisers or supplements. Calving occurs in spring with weaning in November/December, which is when the animals are housed until May. A vet visits at the beginning of each year to take blood samples for health monitoring purposes. Cattle are also vaccinated against Bovine Viral Diarrhoea and receive anthelmintic treatment in the autumn. There had been no notable diseases or animal health incidences during the past five years. The farm was sampled on 06/06/16, 28/06/16, 21/07/16, 11/08/16, 31/08/16, and 24/09/16.

5.2.1.3 Farm C

An organic beef and lamb farm (Soil Association certified) in Angus, UK. The cattle were Aberdeen Angus crossed with Hereford and Galloway bulls. The rotation system sees cattle moved one to three times per day, typically three, on a 90-day rotation cycle. The farm's sheep graze the same land as the cattle, but not at the same time. The ley is a predominantly ryegrass, with the addition of clover and cocksfoot, however, a number of other naturally occurring species are present including thistles and docks.

Minimal intervention is practiced, with no veterinary medicines having been administered during the lifetime of the animals within the study. Additionally, cattle are not housed for any part of the year and calves are allowed to wean naturally. Calving typically occurs during late spring. The farm was sampled on 04/07/16, 18/08/16, 04/10/16 and 07/12/16.

5.2.1.4 Farm X

Farm X is an intensively managed beef and lamb farm in Devon, UK, and is Rothamsted Research's North Wyke Farm Platform. Sampling covered three herds of cattle, although results were pooled as one dataset. Each of the three herds graze in independent systems which differ in pasture, but are otherwise alike (Hatch et al., 2011; Orr et al., 2016), as outlined in Chapter 3. The farm was sampled on 18/07/16, 19/08/16, 21/09/16, and 10/11/16.

5.2.2 Sample collection and analysis

5.2.2.1 Faecal egg counts

On each farm visit ten fresh faecal samples were taken from each herd. Samples were obtained by monitoring cattle until they defecated, at which point samples were immediately collected using a clean plastic spoon, with care taken not to inadvertently incorporate any foreign material into the sample. Samples were stored in screw-top plastic containers in a refrigerated cool box, before being stored at 4°C until being sampled (within 48hrs). No animal was sampled twice during the same visit. Faecal egg counts (FECs) were completed in triplicate, using mini-FLOTAC and fill-FLOTAC devices, in accordance with manufacturer's methods. A 1.34g ml^{-1} flotation solution of zinc sulphate and deionised water (1:1) was created. 45ml of flotation solution was then mixed with 5.0g of faeces, in the fill-FLOTAC device. This mixture was then transferred to the two wells of the mini-FLOTAC and rested for ten minutes. The top of the mini-FLOTAC device was then rotated 90°, skimming off any eggs that floated to the top. Gastrointestinal nematode eggs were then identified and counted under a microscope. Total counted GIN eggs across both wells of the mini-FLOTAC plate were multiplied by 5x to determine epg. Mean FECs across each farm's herd were compared using a Kruskal-Wallis test. This was followed by another Kruskal-Wallis test to determine differences in the burdens of infected animals only. These tests were used to test Hypothesis 1. Egg counts were considered as 'high' if they were above a treatment threshold of 150epg (Soil Association, 2015).

5.2.2.2 Pasture performance

Herbage samples were taken from six fields on each farm visit, providing three replicates of two field types. Three of the fields sampled were those that cattle were to be moved on to for grazing within the next three days, these fields were named as 'return' fields. The other three fields were called 'recent' and were fields that cattle had been grazing within the last three days but had been moved on from. In each field, four herbage samples were taken, sample locations were chosen using a controlled random strategy. Each field was split into quadrants using a laser rangefinder (Shotsaver SLR500) and a random number generator. The length and width of the field were measured and quadrants divided by bisecting lines halfway along each of the measured dimensions. Knowing the size of each quadrant, a random number generator was used to pick X and Y coordinates at which samples were taken. In the

event that coordinates were within five metres of a field boundary or object of significance (such as a tree or trough), a new coordinate would be chosen using the same process. At each sampling location, a 40x40cm quadrat was placed and all herbage above 4 cm removed using horse shears. Collected herbage was placed in a plastic bag, labelled, zip tied, and refrigerated at 4°C. Within one week, samples were oven dried at 65°C until a constant weight. Mean values were taken for each field status (return or recent) of each farm. Total forage dry matter (above 4 cm) of each field status were compared across the study farms using a Kruskal-Wallis test and post-hoc Mann-Whitney U tests. These tests were used to test Hypothesis 1.

5.2.3 Pasture composition

Intra-field replicates of dried herbage samples (as per 5.2.2.2) were composited, providing one sample for each of the six fields sampled on each visit. The composite samples were then ground to < 2mm using a herbage grinder (Retsch SM 300). The composition of herbage samples was analysed by near-infrared spectroscopy (NIRS) using a FOSS NIRS DS2500. Crude protein (CP), neutral detergent fibre (NDF), acid detergent fibre (ADF), and ash were quantified as a proportion of sample dry matter (g kg^{-1}). From those results, it was possible to calculate organic matter concentration as the remainder after the removal ash. Limitations of the equipment meant that directly measuring non-fibre carbohydrates (NFC) and crude lipids (CL) was not possible, however a combined concentration for them both could be calculated (Equation 5.1).

$$NFC + CL\ (g\ kg^{-1}) = 1000 - NDF - CP - ash$$

Equation 5.1 - Calculation of non-fibre carbohydrates (NFC) and crude lipid (CL) concentration.

Results from NIRS were compared in two different manners. The first was a direct comparison of nutritional components between farms, from each farm's pooled data set. This was conducted by comparing each nutritional component, between farms, using one-way ANOVAs with post-hoc Tukey tests. A second analysis was conducted for each mob-grazing farm (Farms A-C), to compare if the nutritional value of herbage varied between forage on 'return' and 'recent' fields. Initially, results from the three farms were pooled into two groups, a 'return' group and a 'recent' group. These were compared using a two-sample *t*-test. If different trends were observed between the farms (for example, one farm saw an increase

whilst others a decrease), then further individual *t*-tests were conducted to investigate these more specifically. These results went towards assessing Hypothesis 3.

5.2.3.1 Soil organic matter

On the final field visit to each farm, soil samples were taken to determine SOM content (% of DM). Four samples were taken from each field, one from each quadrant. Herbage was removed from above the soil using horse shears. Soil cores were then taken to a depth of 8.5cm and stored in sealed plastic bags. Samples were refrigerated during transport (< 24 hr) and then frozen and -20°C until analysis. Soil samples were then oven dried at 65°C to a constant weight (typically 3-5 days), after which stones were removed and samples were ground to a fine power <1mm. Organic matter content (as a percentage of dry matter) was then determined by loss on ignition. Clean porcelain crucibles were furnaced at 450°C for > 3 hr and then cooled in a desiccator. Crucibles were then weighed and 1.0g of dried and ground soil sample was then added, before being placed in a furnace at 360°C for > 6 hr (Salehi et al., 2011). Soil organic matter (% of DM) was calculated as the loss in weight of the crucible and sample, as a proportion of the original 1.0 g sample weight. Soil organic matter content was plotted against FAO definitions for reference (Table 5.1) (Fraters et al., 1993). Soil organic matter contents of each farm were compared using a one-way ANOVA, with a post-hoc Tukey test to identify any differences found. These tests were used to assess Hypothesis 4.

Table 5.1 - Classification of topsoil organic matter content (Fraters et al., 1993).

Topsoil OM (% DM)	Classification
1	Very low
2	Low
3	Low/moderate
4	Moderate
5	Moderate/high
8	High
10	High/very high
14	Very high
30	Organic soils

5.3 Results

5.3.1 Faecal egg counts (GIN)

Faecal egg count results (epg) varied between farms, however, mean values for all farms were below the treatment threshold of 150 epg.

Mob-grazing farms (A-C) tended to have lower GIN burdens than the control farm (X) but this was not significant, likely because of the large variation on the control farm (X). Mean FEC epgs of GIN eggs for the entire herds, across the sampling seasons, were lower on farms A-C (1.75, 0.67, and 1.25) than on farm X (2.82; Figure 5.3). Despite the scale of difference (131% when comparing the mean of means of A-C to X), there was no statistically significant difference between farms, as determined by a Kruskal-Wallis test (H = 6.45, p = 0.092).

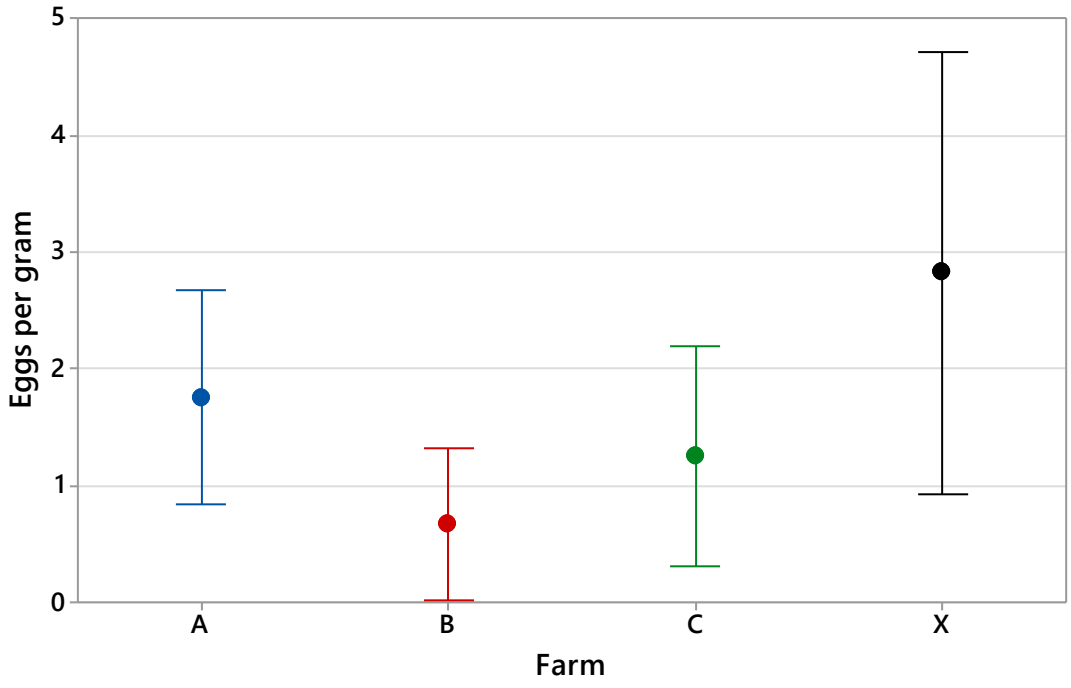


Figure 5.3 - Interval plot of faecal egg count (FEC) results, as GIN eggs per gram (epg). Results represent the entire herd data for each farm. Intervals are standard deviation.

The prevalence of infection varied between farms and was highest on farm A, with 25.0% of faecal samples testing positive for GIN eggs, compared to 8.3%, 13.3%, and 19.3% on farms B, C, and X, respectively. Despite the higher prevalence on farm A, infected animals had the lowest FEC results compared to the other farms. When examining the egg counts only of animals with positive faecal samples (Figure 5.4), this trend persisted (7.00, 8.00, 9.38, and 14.64), however, was still non-significant (H = 3.44, p = 0.328).

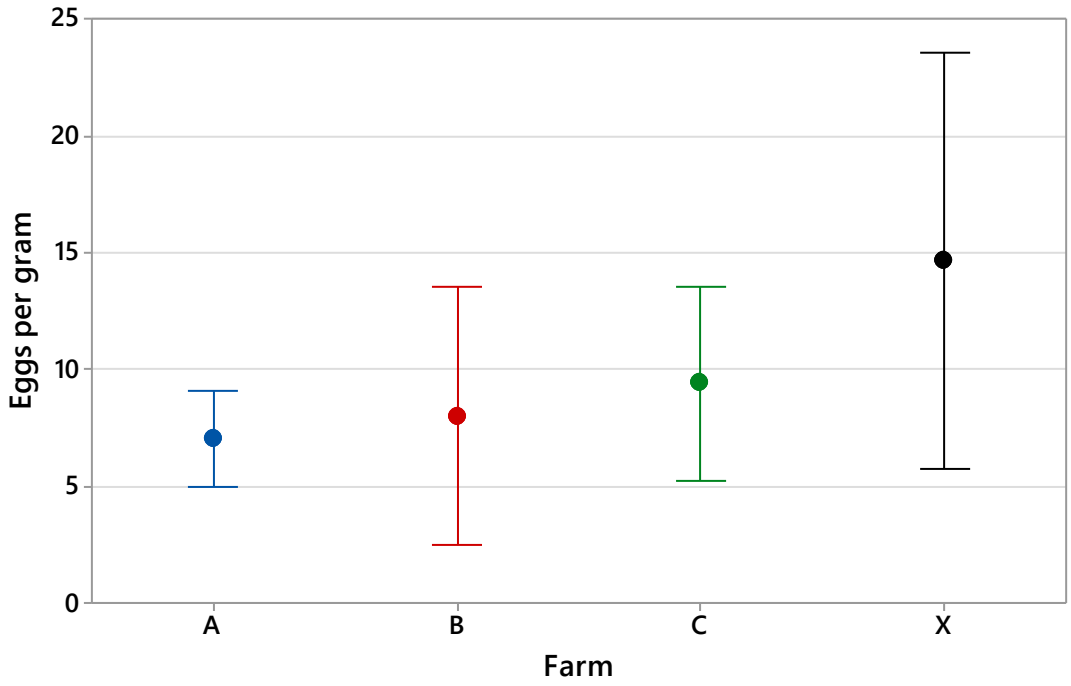


Figure 5.4 - Interval plot of faecal egg count (FEC) results, as GIN eggs per gram (epg). Results represent data for infected individuals only, for each farm. Intervals are standard deviations.

5.3.2 Pasture performance

Fields on farms A-C had higher total masses of dry matter than on farm X, both prior to being grazed and after being grazed (Figure 5.5). A Kruskal-Wallis test found that dry matter (kg ha^{-1}) levels of fields which were about to be grazed were significantly different (H = 74.92, p < 0.0005). Post-hoc Mann-Whitney tests found that 'return' fields on farms A-C had significantly greater forage dry matter than farm X (W = 2351, p = 0.0077, W = 2525, p < 0.0005, W = 1094, p < 0.0005, respectively). The same trend was present for fields which had recently been grazed, with a Kruskal-Wallis test finding a significant difference between farms (H = 19.00, p < 0.0005). Similarly, post-hoc Mann-Whitney tests found that recently grazed fields on farms B and C had significantly greater forage dry matter than on farm X (W = 3259, p = 0.0056, W = 1002, p = 0.0022, respectively). Whilst farm A had a greater mean (9397 vs. 7880) and median (8922 vs. 7813) than farm X, no significant difference was identified by the Mann-Whitney test (W = 2257, p = 0.1575).

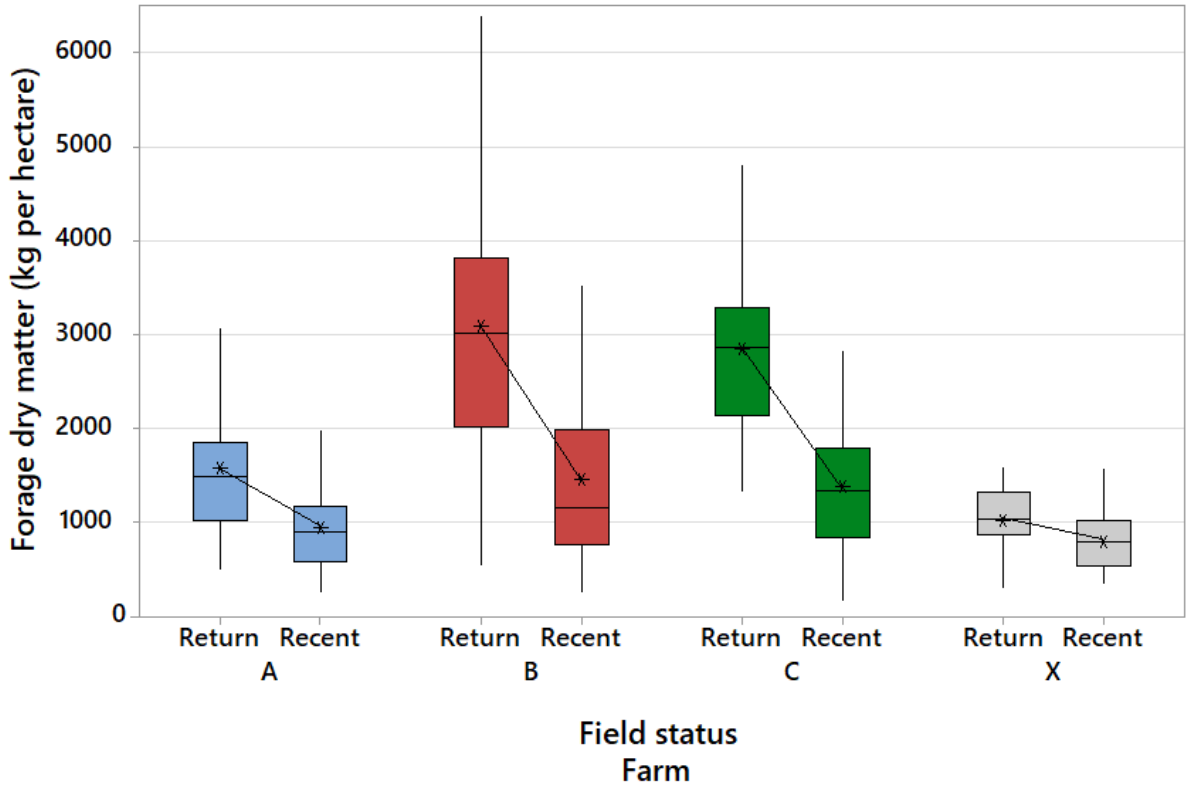


Figure 5.5 - Forage dry matter mass (kg per hectare) for the four farms. "Return" refers to fields which are at the end of a fallow period, between grazing. "Recent" refers to fields which cattle were grazing, but have come off, within the last three days. Asterisks represent means.

5.3.3 Forage nutrition

5.3.3.1 Direct farm comparisons

ANOVA and Tukey test results found statistically significant differences, between farms, for every nutritional component analysed.

Organic matter concentrations were relatively similar between farms (Figure 5.6), but Farm B having a significantly higher concentration than Farms A, C, and X (F = 8.92, p < 0.0005). Despite not being significantly different, Farm X had the lowest mean of 905.6 g $kg^{-1,}$ compared to Farms A and C, which had similar concentrations of 927.7 and 928.7 g kg^{-1} respectively.

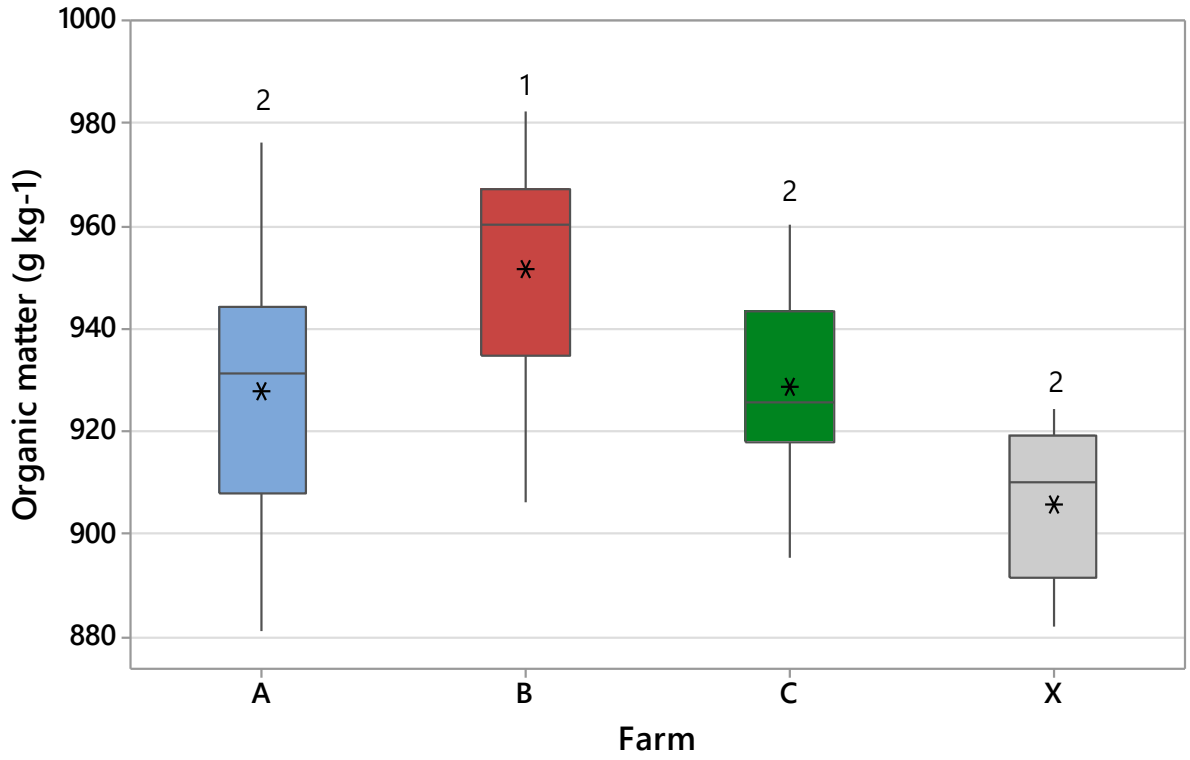


Figure 5.6 - Boxplots showing the distribution of organic matter (OM) content (g kg^{-1}) of forages recovered from each farm. Boxplots that do not share one or more similar number above them are significantly different. Asterisks represent mean values.

Ash concentrations are calculated as the inverse to organic matter concentrations and thus showed inverse trends (Figure 5.7) with Farm B have significantly lower ash concentrations than the other farms (F = 8.92, p < 0.0005). As with OM results, there is notable overlap between farms both, between quartiles 1 and 3 (box) and the data range (whiskers).

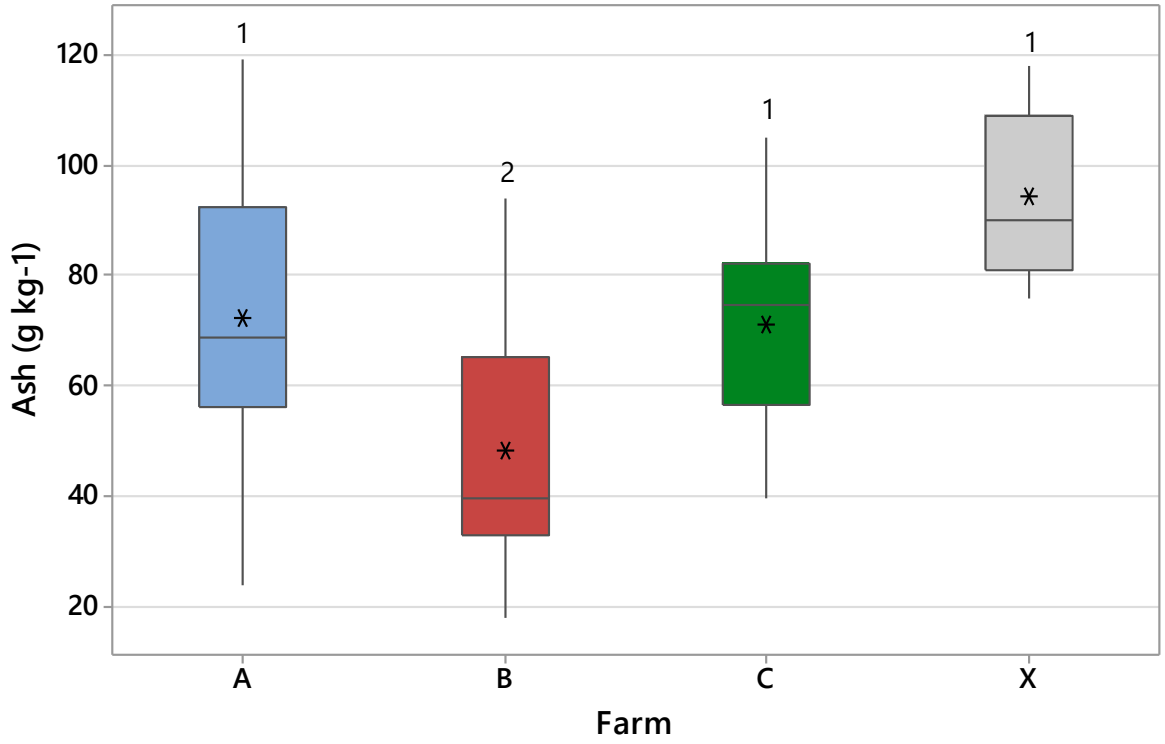


Figure 5.7 - Boxplots showing the distribution of ash content (g kg^{-1}) of forages recovered from each farm. Boxplots that do not share one or more similar number above them are significantly different. Asterisks represent mean values.

Neutral detergent fibre concentrations varied significantly between farms (Figure 5.8; F = 12.11, $p < 0.0005$) with Farm B having the highest mean concentration (627.2 g kg^{-1}), Farms A and C lying in the middle with similar concentrations (570.2 and 567.7 g kg^{-1}, respectively), and with Farm X having the lowest mean (477.0 g kg^{-1}). Notably, Farm A had a very high spread of results with a standard deviation of 74.3, compared to 56.4, 45.4, and 28.6, as observed in farms B, C, and X respectively. This spread also included both the highest and lowest values of all data sets.

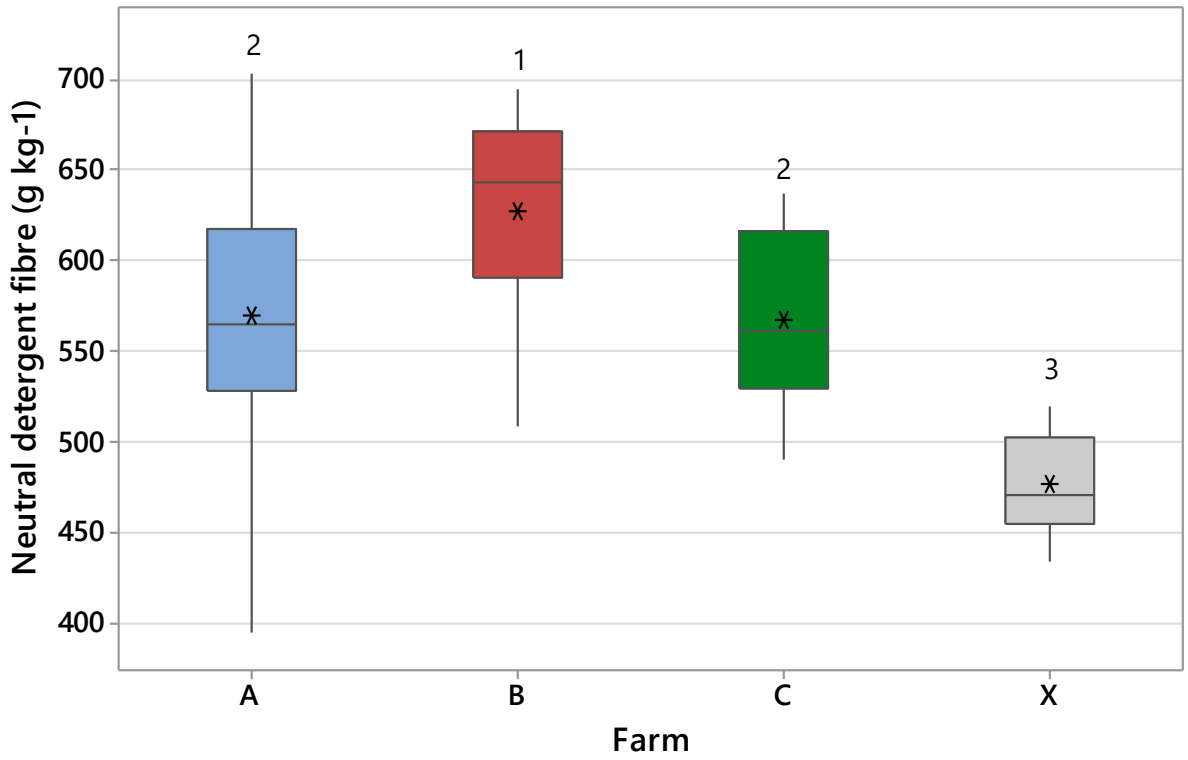


Figure 5.8 - Boxplots showing the distribution of neutral detergent fibre (NDF) content (g kg^{-1}) of forages recovered from each farm. Boxplots that do not share one or more similar number above them are significantly different. Asterisks represent mean values.

Acid detergent fibre, a component of NDF also varied significantly between farms (Figure 5.9) (F = 48.13, p < 0.0005) with a clear grouping visible for Farms A, B, and C with average values around 375 to 400 g kg^{-1} and the range boxes considerably overlapping. This is in contrast to Farm X where ADF concentrations were significantly lower than on Farms A to C, averaging at approximately 250 g kg^{-1} and with the greatest variety of all samples, but one which is relatively evenly spread as represented by the large range box and small whiskers.

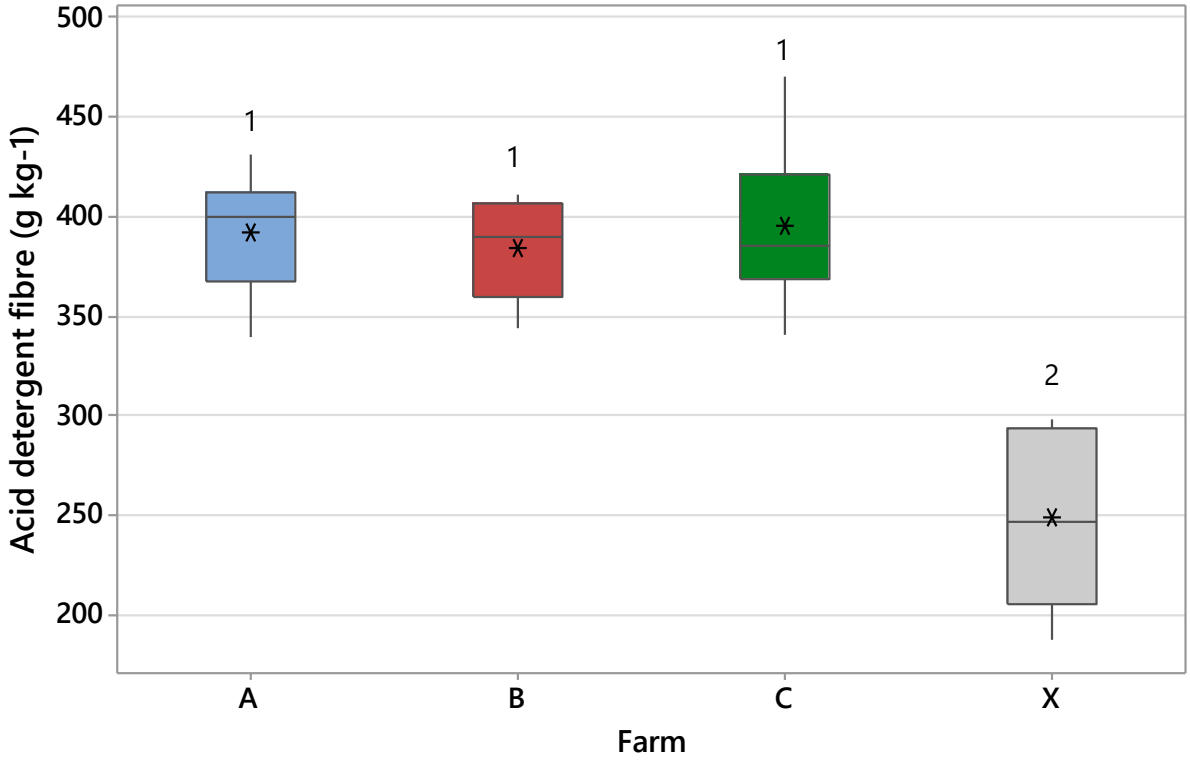


Figure 5.9 - Boxplots showing the distribution of acid detergent fibre (ADF) content (g kg^{-1}) of forages recovered from each farm. Boxplots that do not share one or more similar number above them are significantly different. Asterisks represent mean values.

Crude protein concentrations of forage from the different farms yielded the most complex of the nutritional results (Figure 5.10) with three levels of statistical difference ($F = 7.50$, $p < 0.0005$). Concentrations varied greatly from examples of less than 30 g kg^{-1} on Farm B to over 250 g kg^{-1} on Farm A. This pattern was similar to that observed for organic matter content (and thus inverse to that observed for ash).

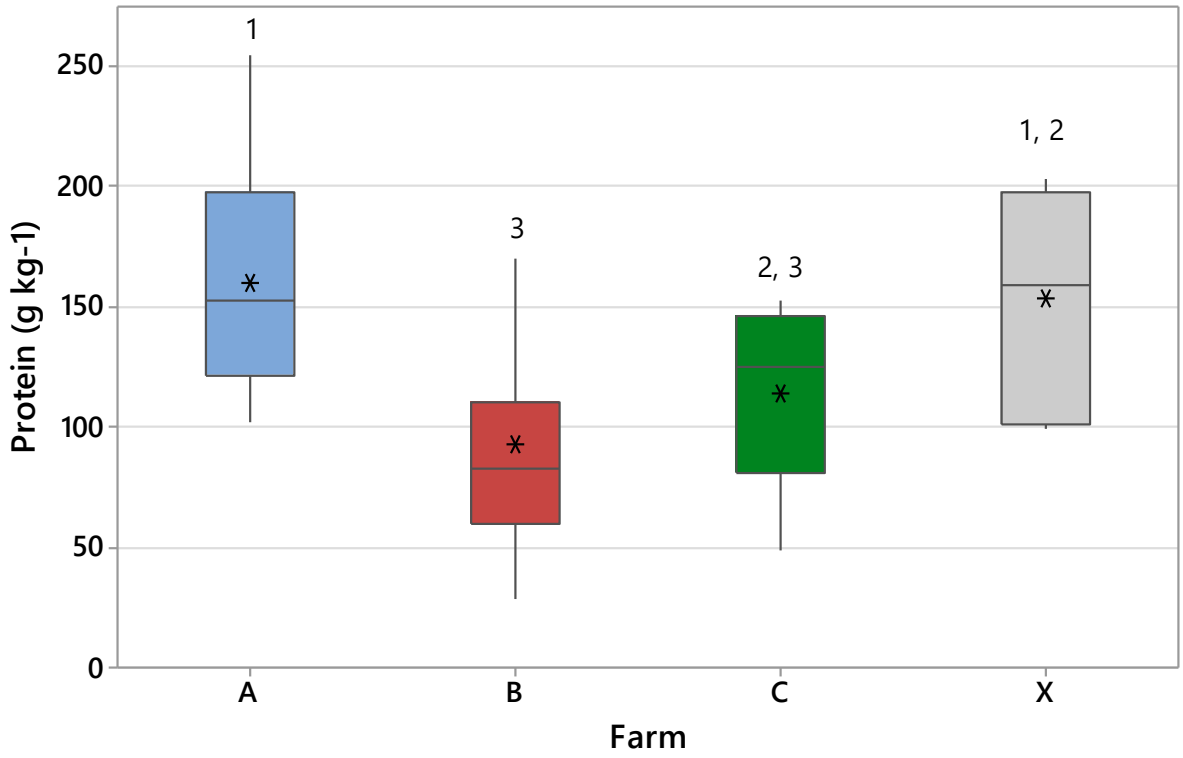


Figure 5.10 - Boxplots showing the distribution of protein (CP) content (g kg^{-1}) of forages recovered from each farm. Boxplots that do not share one or more similar number above them are significantly different. Asterisks represent mean values.

Combined concentrations of NFC and CL were significantly different between farms (Figure 5.11) (F = 4.80, p = 0.005). Whilst farms A and X were significantly different to one another, Farms B and C were both not significantly different to any of the other farms. Despite the seemingly minor differences seen here, compared to the other nutritional components analysed, mean concentrations from Farm X (275.4 g kg^{-1}) were 39.4% greater than the mean of Farm A (197.6 g kg^{-1}).

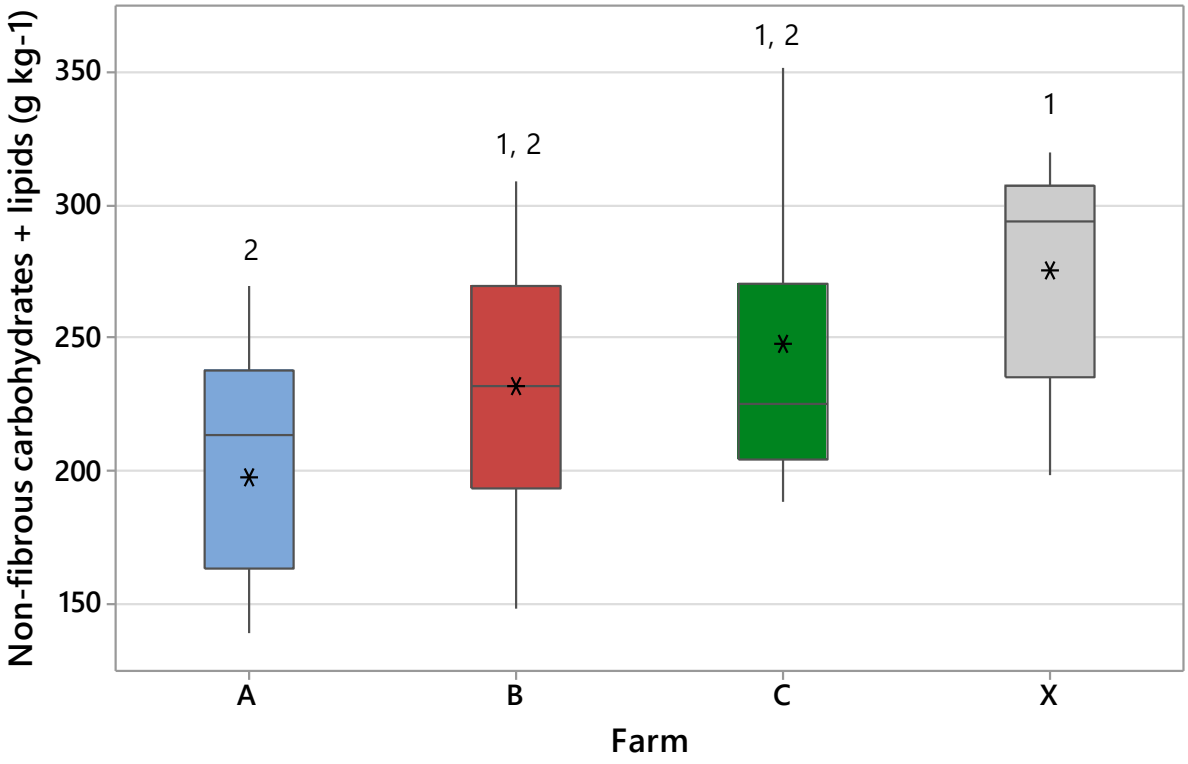


Figure 5.11 - Boxplots showing the distribution of combined non-fibre carbohydrate (NFC) and crude lipid (CL) content (g kg^{-1}) of forages recovered from each farm. Boxplots that do not share one or more similar number above them are significantly different. Asterisks represent mean values.

5.3.3.2 Field status comparison

Comparisons of forage from fields which cattle were returning to, with those which had been recently grazed, found significant differences in a number of components.

Forage organic matter (Figure 5.12) concentrations did not significantly differ between 'return' and 'recent' fields (t = 0.03, p = 0.979). Farm A saw a relatively minor decrease in OM between 'return' and 'recent' fields, from 930.6 to 924.8 g kg^{-1}, whilst Farm B saw a larger decrease (959.3 to 945.3 g kg^{-1}) and Farm C saw an increase (922.9 to 939.3 g kg^{-1}), the largest observed difference.

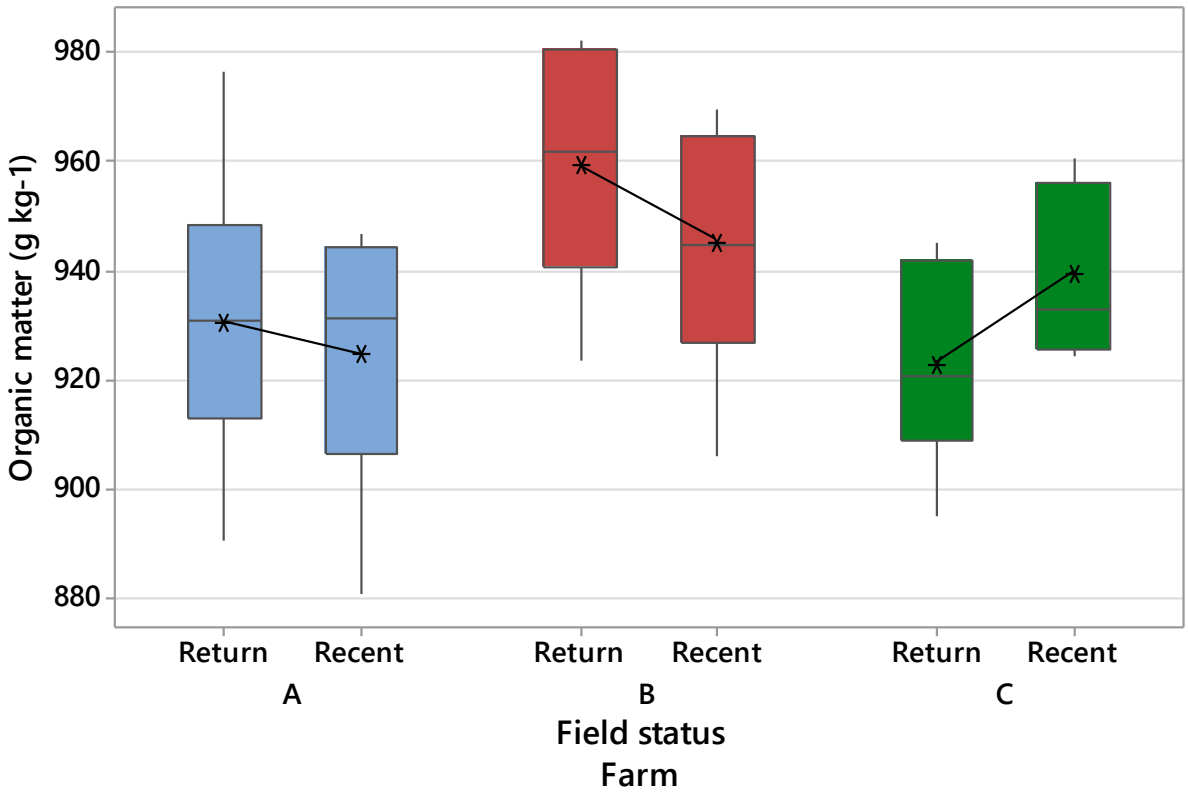


Figure 5.12 - Differences in organic matter (OM) concentration (g kg^{-1}) between 'return' and 'recent' fields on mob-grazing farms (Farms A-C). Asterisks represent mean.

No significant difference was found between forage NDF concentrations on fields that cattle were returning to (Figure 5.13), compared to fields that cattle had recently grazed (t = 1.08, p = 0.287). Whilst both field types yielded relatively similar concentrations for Farms A and B, this was not the case for Farm C, where 'return' fields had mean NDF concentrations 540.5 g kg^{-1} compared to 616.4 g kg^{-1} on recently grazed fields. When analysing Farm C alone, the difference was significant (t = 5.50, p < 0.0005).

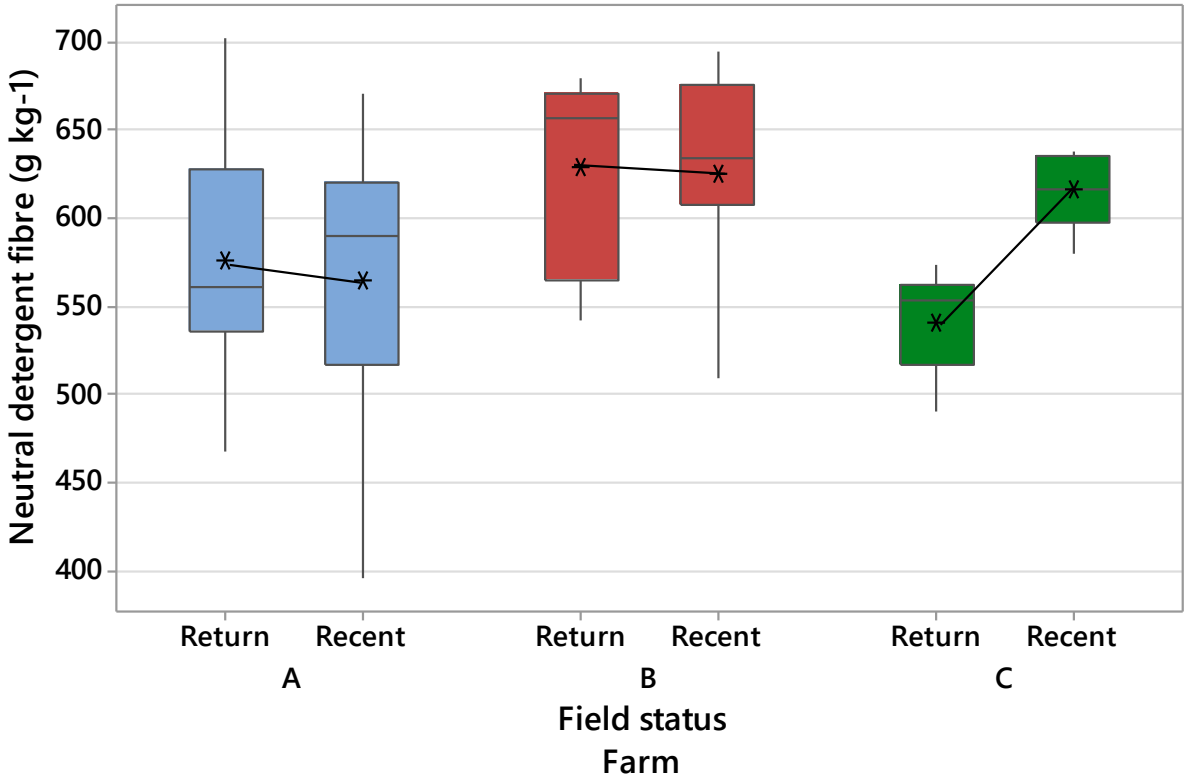


Figure 5.13 - Differences in neutral detergent fibre (NDF) concentration (g kg^{-1}) between 'return' and 'recent' fields on mob-grazing farms (Farms A-C). Asterisks represent mean.

Whilst NDF concentrations were not significantly different between field types, ADF concentrations were (Figure 5.14; t = 2.87, p = 0.007). However, whilst increases in the proportion of forage ADF after grazing were seen on all farms, this was minor for Farm A, and moderate for Farm B. Farm C showed a far greater increase, from a mean of 373.2 to 437.8 g kg^{-1}. When looking at farms in isolation, the significant difference remains for Farm C (t = 5.08, p = 0.004), but is not present for Farms A and B (respectively: t = 0.18, p = 0.857, t = *1.40*, p = 0.198).

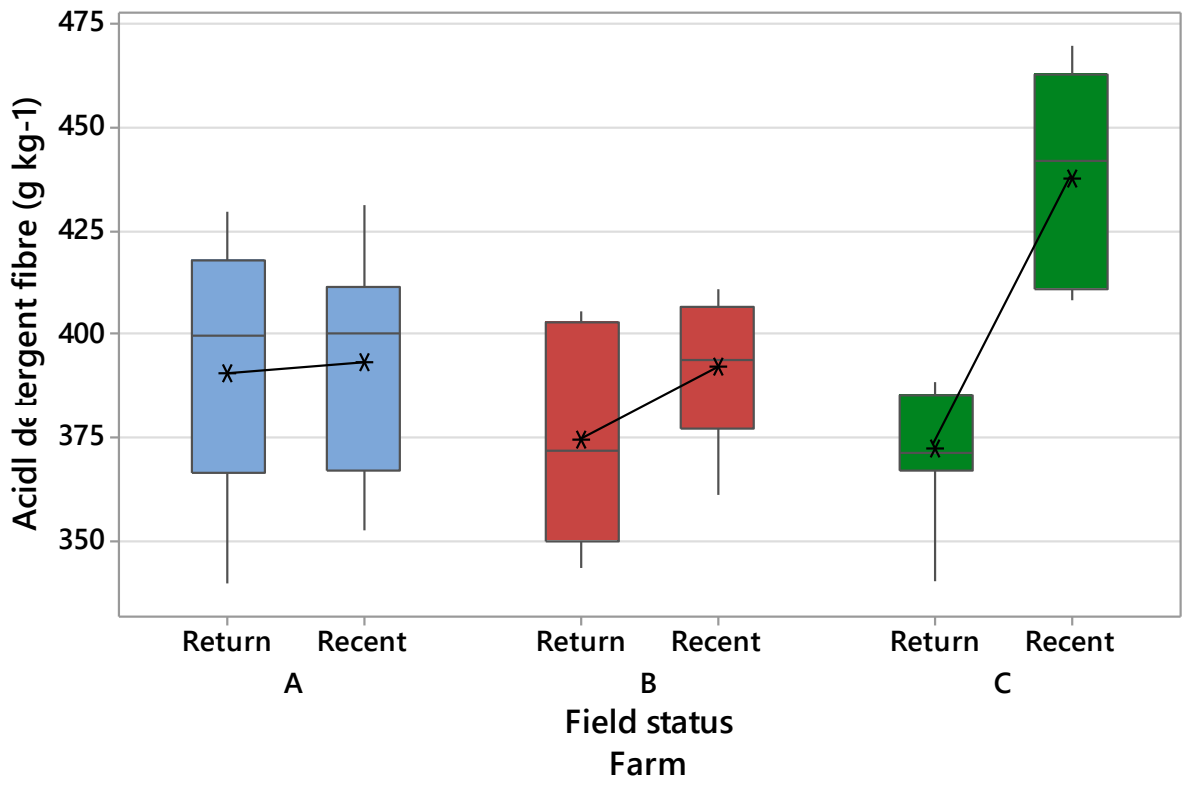


Figure 5.14 - Differences in acid detergent fibre (ADF) concentration (g kg^{-1}) between 'return' and 'recent' fields on mob-grazing farms (Farms A-C). Asterisks represent mean.

Crude protein concentrations varied significantly between field types (Figure 5.15; $t = 1.96$, $p = 0.029$). Differences, between field types, were statistically significant for Farm B ($t = 2.80$, $p = 0.021$) but not for Farm A ($t = 1.36$, $p = 0.098$) (although close) and Farm C ($t = 0.10$, $p = 0.920$). In all instances the proportion of CP in forage was lower in pastures that had recently been grazed, in comparison to those which had just finished a fallow period.

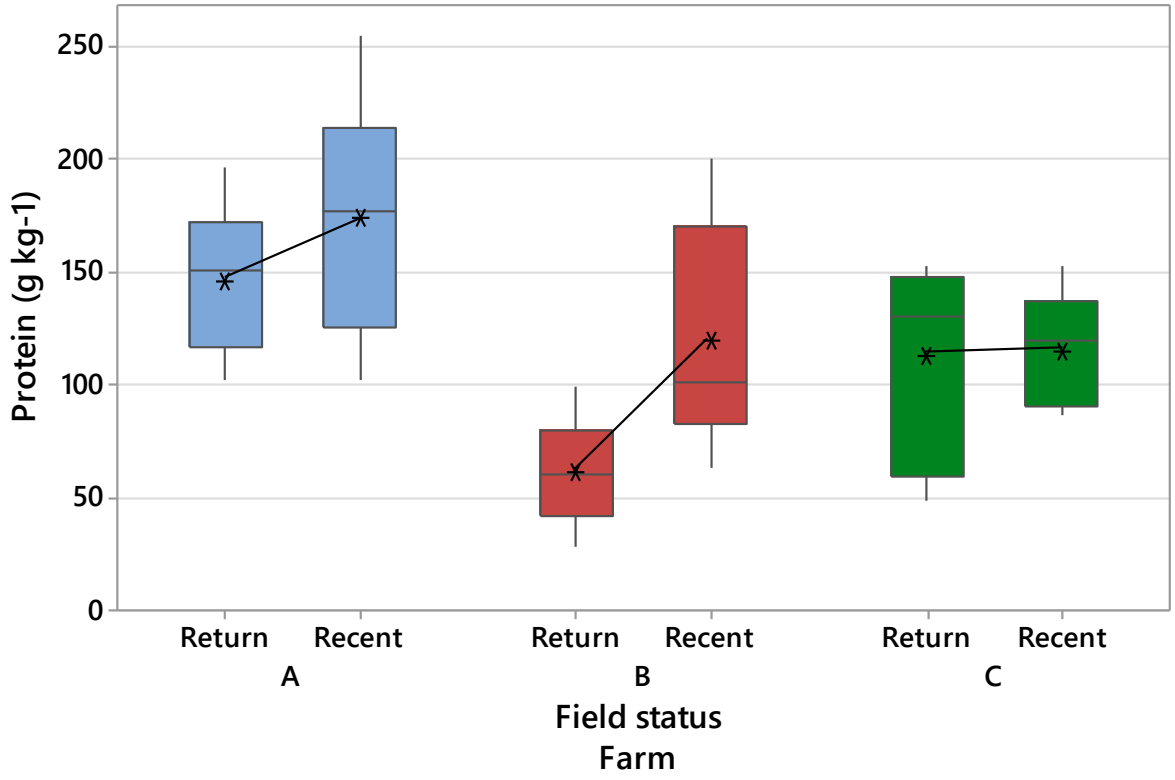


Figure 5.15 - Differences in crude protein (CP) concentration (g kg^{-1}) between 'return' and 'recent' fields on mob-grazing farms (Farms A-C). Asterisks represent mean.

Statistically significant differences were found, between field types, for combined concentrations of non-fibre carbohydrates and lipids (t = 3.19, p = 0.003; Figure 5.16). Across the three farms, concentrations of combined carbohydrates and lipids were greater in fields where cattle were returning to, compared to those recently grazed.

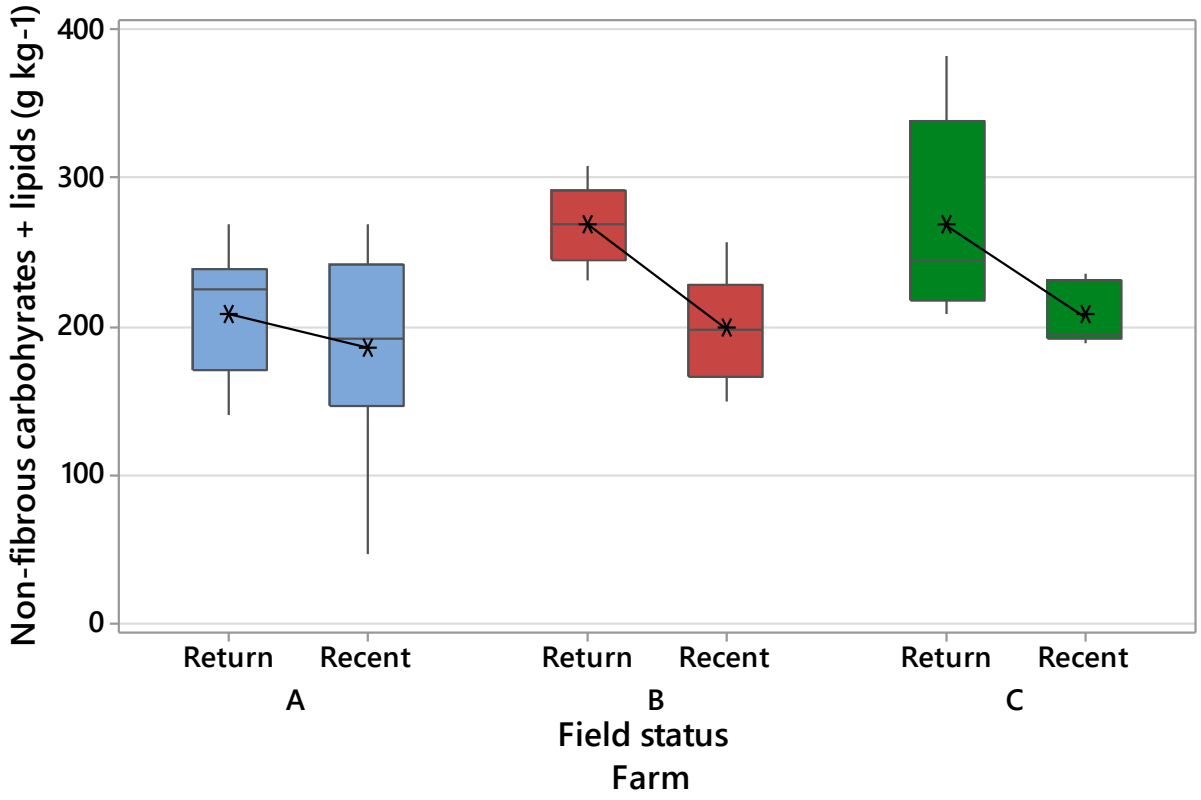


Figure 5.16 - Differences in combined carbohydrate and lipid concentration (g kg^{-1}) between 'return' and 'recent' fields on mob-grazing farms (Farms A-C). Asterisks represent mean.

5.3.4 Soil organic matter

A Grubb's outlier test found one outlying result (p = 0.004) across all farms, taken from Farm C, with a recorded organic matter content of 33.4%. This outlier was removed prior to analysis. Soil organic matter, for all farms, was classified as high/very high (Figure 5.17). The highest being found on Farm A, with a mean of 13.4%, followed by C, X, and B (11.5, 10.2, and 10.0%, respectively). The variation was particularly high in Farm C, with a difference between Q1 and Q3 of 5.8 percentage points and total range of 21.1 percentage points. A one-way ANOVA found statistically significant differences between soil organic matter content across the four farms (F = 13.55, p < 0.0005). A post-hoc Tukey test identified those differences (Figure 5.17) showing that levels on Farm A were significantly higher than on all other farms and that those on Farm C were significantly higher than on Farm X.

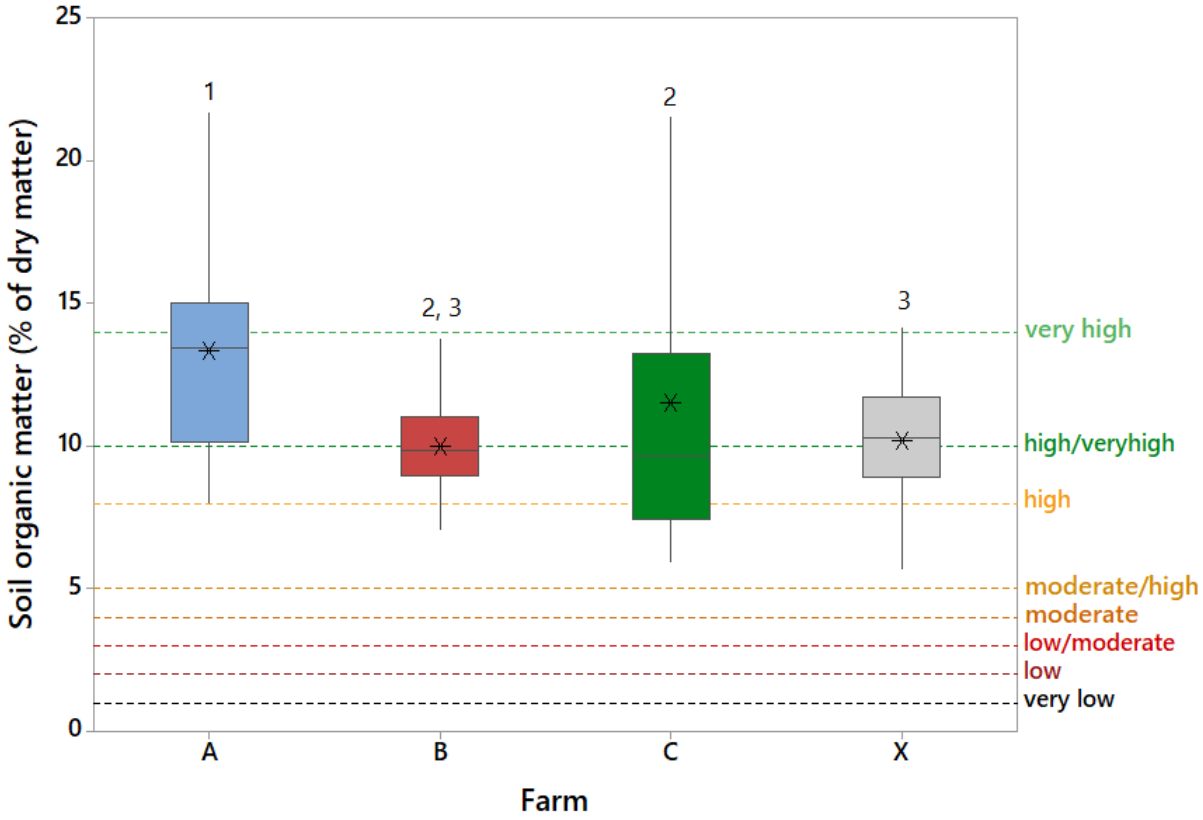


Figure 5.17 - Boxplots of soil organic matter (as a percentage of soil dry matter) across the four farm sites. Asterisks represent sample mean. Numbers above boxplots represent statistical groupings, as defined by a Tukey test. Groups sharing the same number are not significantly different. Reference lines on soil organic matter classification are providing in line with FAO definitions (Fraters et al., 1993).

5.4 Discussion

The conceptual justification for mob-grazing and its benefits are founded on basic scientific principles. However, there is very little applied research to support this. The evidence found throughout this experiment is, in all instances, either supportive or neutral to those justifications.

5.4.1 Faecal egg counts

Faecal egg count results found parasite burdens to be lower on mob-grazing farms than on the control. It is also notable that Farm X had high variability in results. Within agricultural systems, variation and inconsistencies can be problematic and mean that developing an effective parasite control strategy is more difficult. These results go some way to supporting the hypothesis that the rotational nature of mob-grazing can be used as a method of GIN control. The grazing rotations implemented on Farms A-C, with cattle being moved every three days or less, mean that GIN eggs deposited in faeces will become infective after cattle have moved to new pasture. The length of the rotation cycles (60-90 days) mean that cattle are also not on the pasture during times of peak infectivity (typically 1-3 weeks). Between that time and the time cattle return to pasture, the infectivity of the pasture will have continually decreased as GINs die or are removed. This potential 'breaking' of the GIN life-cycle may be part of the reason why such low egg counts were observed.

Although not measured, the apparent high biodiversity of Farms A-C, facilitated by the lack of anthelmintics, sward diversity, and soil quality may also have contributed to the low egg counts. Organisms, such as soil invertebrates, fungi, bacteria, and predatory nematodes, are known to act as biological controls for GINs (Bryan, 1976; Fincher, 1973, 1975; Larsen et al., 1994; Larsen, 2000; Waller, 2006; Waller and Faedo, 1996; Waller and Larsen, 1993). If this is the case, the system may be self-perpetuating as a reduction in GINs, due to these biological process, which lessens the need for anthelmintics by reducing the risk of outbreaks.

Whilst Farm A had the greatest proportion of infected animals, the mean epg of those animals was the lowest of all farms. This suggests the possibility that the animals are resilient and able to tolerate low or moderate levels of infection. Farm A also had the least variation in FEC results between infected individuals. The location of farm A (Cornwall, UK) is particularly high risk for GIN infections due to the relatively warmer and wetter climate. Combining these

strands of evidence suggests that individuals on Farm A have a relatively high GIN challenge, but have consequently developed tolerance mechanisms. The practical benefit of this would be that, whilst GIN risk is persistent, the chance of losses from GIN infections is relatively low. A herd level resistance or resilience to GINs and other parasites could yield long-term benefits to herd health and farm sustainability. The lack of anthelmintics used on the mob-grazing farms means that the local GIN population is likely to be susceptible to anthelmintics, providing the farms with the confidence that anthelmintics can be effectively applied in the event of an outbreak.

The complexity of the topic means it is unlikely that one factor is the driver of the observed egg counts. A more realistic scenario is that all of such factors over time have had a cumulative impact on local epidemiology by exerting pressures on GINs throughout all stages of their life-cycle. This is in stark contrast to anthelmintic-orientated controls, which consistently use one method of control that focuses on one part of the life-cycle. Such a precise selection pressure can drive the rapid development of resistance. The potential of mob-grazing for GIN control highlights the need for comprehensive integrated control strategies that utilise all of the resources available, including natural biological controls and our knowledge of GIN lifecycles. Whilst the evidence supports mob-grazing as a method of livestock management, further work is needed to investigate epidemiological factors in more detail, specifically to identify the drivers and mechanisms involved.

The principles of anthelmintic control are not unique, and many are broadly applicable to the control of other prevalent diseases. For example, the degradation of dung by invertebrates has been linked to the removal of TB (Phillips et al., 2003). This is a prime example of the ethos of holistic farming, promoting the underlying biological process of a system and employing those benefits for agricultural gain.

5.4.2 Pasture performance

There was a notable and statistically significant difference in dry matter (kg ha^{-1}) between the farms, particularly between Farms A-C and Farm X. The mob-grazing farms had greater dry matter on pasture when cattle were put onto it and when they were taken off, leaving a greater residual. In between being grazed, herbage on Farms A-C had significant growth of 67%, 112%, and 207%, respectively, whilst Farm X had growth of just 29%. Even if percentage

increases were the same, the greater residual herbage levels of Farms A-C would result in a greater total herbage gain than Farm X. These results support the idea that mob-grazing can be used to control foraging behaviour to the benefit of pasture productivity.

5.4.3 Forage nutrition

5.4.3.1 Direct farm comparisons

The multi-factorial nature of forage nutrition analysis means it is generally not possible to say if any particular forage is 'better' than any other. OM results are a broad indicator as it represents the portion of forage which can potentially be utilised by the animal. OM concentrations were significantly greater on Farm B than on Farms A, C, and X, which were all statistically similar. However, a closer look at the composition of that OM component reveals a much more complex picture.

NDF and ADF yielded particularly interesting results. NDF is typically associated with forage digestibility and is therefore considered a beneficial trait (Mertens and Ely, 1979; Oba and Allen, 1999). Farms A-C had significantly greater NDF concentrations than Farm X. Whilst this may seem like an obvious benefit in favour of the mob-grazing farms when looking deeper into the nutritional quality, this is not necessarily the case. A primary component of NDF is ADF, which is considered highly indigestible and, therefore, has a negative association with nutritional quality. ADF levels were significantly higher in Farms A-C than in Farm X. It is also important to consider that lower levels of a particular nutrient can mean that another nutrient is more abundant. Farm X had the highest levels of NFC and lipids. However, this was only significantly greater than Farm A and not B, and C. NFC is a highly valuable nutrient, which is energy-rich and can aid digestibility of NDF (Arroquy et al., 2005; Haddad and Grant, 2000). Whilst NFC and lipids were combined due to the limitations of NIRS technology, from results gathered in Chapter 3 we can estimate the lipids values would likely range from 10-70 g kg^{-1}. Protein concentrations yielded complex results and werenoticeably highest on Farm A, despite not being significantly different to Farm X. Protein is important and relates positively with growth and final carcass weight, it is also especially important during times of stress (Beaty et al., 1994). The lowest protein concentrations were found on Farm B, which also does not house cattle over winter, this has the potential to cause problems during particularly harsh winters and could reduce animal performance. Nevertheless, this is not a characteristic

of mob-grazing itself. It should also be noted that legumes are particularly protein-rich and can also fix nitrogen from the environment, this may be particularly important to organic farming which does not rely on external animal nutrient and fertiliser inputs.

The composition of forages could be considered of similar quality between the farms. Whilst significant differences were found for certain components, the true implication of these is unknown and circumstantial. For example, dry matter consumption can be variable on different pastures and could not be considered within this study.

5.4.3.2 Field status comparison

Comparing the forage nutritional composition of different statuses of fields yielded a number of interesting results. The statistically significant differences seen could be down to a number of different factors (1) That cattle are selectively grazing in favour of forage with particular nutrients (2) As forage grows, the proportions of nutrients within it alter, for example, more lignin to provide stability (3) The nutritional composition of taller forages varies from those lower down, meaning that cattle are grazing nutrients disproportionately to the fields average composition. In reality, it is likely that all of these factors were at play. During field visits, cattle were observed to be actively selecting particular plants, such as flower heads from chicory. Equally, cattle typically graze the top portion of herbage.

One of the most notable differences was for combined carbohydrate and lipid concentrations which, in all instances were higher in 'return' fields than in 'recent' fields. Whilst, from the data, it is not possible to determine what the precise ratio of carbohydrates to lipids are, from evidence gathered in Chapter 2 it could be inferred that this is overwhelmingly carbohydrate. Therefore, cattle entering a fresh grazing cell are disproportionately consuming carbohydrate, which is a highly digestible and energy-rich resource (Hoover and Stokes, 1991). This result also infers that, during the fallow period between grazing, the proportion of carbohydrate in the forage is increasing. Whilst more specific evidence would be needed to confirm this observation, this is likely to be beneficial for cattle performance, and therefore this may represent a production benefit of mob-grazing, compared to conventional systems which have shorter fallow periods. However, Farms A-C had lower concentrations of carbohydrates in general, and the highest mean level of those farms at any point (Farm C, return, 269.4 g kg^{-1}) was slightly less than the mean for Farm X (275.4 g kg^{-1}). Carbohydrate results tie in well

with ADF results, which showed an increase in concentrations from 'return' to 'recent' fields. ADF is highly indigestible and, as a result, is inversely linked to forage digestibility. The increases seen suggest that ADF-rich feeds are not being consumed. Whilst this is beneficial on the mob-grazing farms, it is noted that the forages on these farms had a higher proportion of ADF than Farm X in the first place. Without further specific research into this one factor, it is not possible to assess if the ADF consumption varies between any of the study farms.

The evidence that pasture growth changes in the nutritional composition of forages is very important when considering mob-grazing as a grazing technique. This importance stems from the large differences in dry matter levels seen between fields that are about to be grazed and those that have recently been grazed, differences far greater than those on the control farm (Farm X). As a result, greater fluctuations in the nutritional profiles of forages would be expected on mob-grazing farms and is a key consideration for the weight gain and health of cattle.

5.4.4 Soil organic matter

All farms had soil organic matter contents classified as high/very high (Fraters et al., 1993), but showed significant differences between each other. Within the studied farms, Farm A had the highest soil organic matter content. Climate is one potential driver of this, with Farm A being the warmer and wetter than Farms B and C. Findings by Burke et al. (1989) showed that precipitation was positively linked with soil organic matter, however, the author also commented that high soil clay content could also lead to high soil organic matter. Farm A sits on mudstone, which has a high clay content and therefore may be somewhat responsible for the high organic matter observed. However, Farm X is also based on mudstone, yet had the lowest soil organic matter content, although that clay is deeper (British Geological Survey, n.d.). The higher forage biomass of Farms A-C, compared to X, may promote soil organic matter content due to the more extensive and diverse root systems required to support this flora. This high biomass could also have a stabilising effect on the O-horizon and topsoil by creating a microclimate, trapping moisture and reducing temperature fluctuations, creating a more stable soil environment; this is a key area for future research. Whilst the significant differences observed are not dramatic their importance should not be understated. Soil is the

biological foundation of pasture systems and is essential for their long-term productivity and resilience.

5.4.5 Confounding factors

It is important to note a number of confounding factors which may have influenced results. Firstly, the farms that volunteered within this study run by very conscientious farmers who engage with the advances in agricultural science. Observed differences or perceived 'benefits' may be relatively difficult for a typical individual to achieve. Secondly, Farms A-C were organic; Farm X was not. This means that any differences shown may be more indicative of organic farms, as opposed to mob-grazing farms. Notably, there are strategies utilised by many mob-grazing and organic farms, but which are not a requirement of either management style. Such strategies may have indirect impacts on the metrics covered in this study. Examples of this include high pasture diversity and wildlife refugia through diverse sward compositions.

Forage analysis utilised employed technology, which utilised calibrations based on wet chemistry values. The NIRS calibrations used were not specifically designed for the diverse forages collected within this study, and therefore true values might vary slightly from those gathered here. Nevertheless, the true relative values would likely be comparable in terms of scale and variation.

5.4.6 Future research

Results support and justify further research into mob-grazing. Whilst a number of striking differences can be observed, the precise drivers and mechanisms of these differences are unknown, as is the influence of confounding factors. It is also necessary to gain a more detailed understanding of how the method can be applied and the benefits and risks that may be associated with it. This study was somewhat limited in resources, and therefore there is scope to expand the variables considered and to look at them in significantly more detail. A key aspect of this would be to conduct a more longitudinal study with more regular sampling, perhaps with remote automated sampling equipment.

Soil characteristics could be looked at in far more detail, especially given the high organic matter levels observed in this study. More detailed study would allow for the assessment of factors generating high soil organic matter levels and the benefits it is having to the system.

Microbial biomass and community structure would be an important measurement and would provide information regarding the different processes within the soil; it could also identify species of nematophagous fungi which may be contributing to the low parasite egg counts. Compaction is another important factor. The nature of mob-grazing means that the regularity and distribution of hoof-fall is greatly varied. Whilst cattle are within a cell compaction is high for a very short period, but then a long rest period is allowed. The net impact of this is unknown in relation to this grazing practice.

A striking characteristic of the mob-grazing farms was the species diversity and richness of pasture, albeit anecdotal. However, this is not necessarily due to the practice of mob-grazing, but may be down to other management decisions, although the two may be somewhat related. Pasture diversity has the potential to increase system stability and enhance local biological communities, such as those in the soil. Self-medication of herbivorous hosts is often overlooked, but can significantly influence parasite epidemiology and, in these species-rich environments, could be a driver of low parasite burdens. (Villalba and Landau, 2012). Another key aspect of forage that needs examination is plant biomass in relation to the length that herbage is allowed to grow to. As grasses and other flora grow, their productivity changes over time, as does their nutritional composition. The impacts of these differences need assessment and should be considered in the context of the other benefits of high herbage biomass, such as soil moisture retention and health. These differences could manifest themselves across various aspects of both system and animal health.

There are three primary ways through which evidence could be gained to further investigate mob-grazing, addressing the points outlined. Similar to this study, gaining evidence from farm case-studies would allow for realistic insights to be made into the technique. This would come from farms already practising mob-grazing, analogous farms not practising mob-grazing (as a control) and from farms who are switching to mob-grazing. Controlled field trials could be used to compliment this work, by removing confounding factors which may have been present in case studies. Not only would this back up any case study findings, but it would also highlight areas where future research may be needed. A final method is through computer modelling, such as pasture productivity models and epidemiological models. An example of this is the Gloworm-FL model (Rose et al., 2015), which could be adapted to assess the impact of different rotational grazing strategies towards parasite development and infection-risk.

Modelling would provide a theoretical backing to findings, whilst also enabling the study of factors which may be too complex or impractical to assess in a case study or field trial scenario.

5.5 Conclusion

This research supports the justification of furthering research into mob-grazing as a method for pasture-based beef production systems and potentially for the production of other grazing livestock. In particular, positive impacts have been observed towards soil organic matter content, parasite burdens, and pasture performance. These findings are line with anecdotal reports on the topic. Long fallow periods between grazing seem to be beneficial for the improvement of pasture composition, increasing the proportion of NFC available for consumption, whilst decreasing ADF. However, the levels observed in the mob-grazing farms cannot be considered any better than those in the control farm, without significant further study focused on this individual factor. The benefits observed are at a system level and support the idea of a self-propagating system that utilises and exploits the underlying biotic processes of the system. Whilst the evidence broadly supports mob-grazing, the precise drivers are unknown and therefore confounding factors may be an influence. Nevertheless, results provide baseline evidence supporting mob-grazing, however, substantial further work is necessary to deepen and broaden our understanding of the technique, its implications, and how it can best be applied. Such work requires a multi-disciplinary approach in both real and controlled settings. Included within this should be an in-depth analysis of the economic impact and sustainability of mob-grazing systems. If mob-grazing can yield benefits towards system productivity and if the underlying mechanisms can be understood, mob-grazing and other intensive rotational techniques have the potential to become valuable strategies for the improvement of livestock farming in the UK and worldwide.

Conclusion

The outcomes and conclusions of this thesis represent an original contribution to the field of veterinary and agricultural science. The original research presented, supported by the reviewed literature, expands the understanding of the dynamics of beef production systems in the UK. In particular, how this related to scientific capabilities can be more effectively applied in an agricultural context. This was achieved through the utilisation and adaptation of existing methodologies and the development of new and original techniques. This multi-disciplinary approach has brought together broad system factors to investigate how they interact and can be managed more effectively. In addition to the novel findings, the research highlights areas where further research is necessary and how that can be achieved.

The results gathered through this study accept the overarching hypothesis that intentional management of dung as a critical resource on-farm has multiple benefits that improve the resilience of beef production systems.

6.1 A hypothetical farm

The evidence gathered can be utilised to form a hypothetical picture of a more efficient and sustainable beef production system, based on the studied strategies and system factors.

6.1.1 Sward diversity

A high-diversity sward can yield both long- and short-term benefits to grazing systems. Such sward may include high-yielding grasses (e.g. ryegrass), whilst including a mix of legumes and herbs (e.g. clover and chicory). The nitrogen-fixing capabilities of legumes will help to reduce the need for external fertiliser inputs (Groffman et al., 1987; Ledgard and Steele, 1992), whilst herbs will diversify cattle nutrients. Bioactive herbs can reduce the need for external feeds during finishing, can be high yielding, and can also act medicinally, reducing the need for anthelmintics (Githiori et al., 2006; Peña-Espinoza et al., 2016; Sandoval-Castro et al., 2012; Williams et al., 2016). A less immediately noticeable impact of a diverse sward is system stability, through ensuring that species are present which are able to flourish in different conditions (Brougham, 1960). This diversity also provides a wider variety of resources for organisms, such as invertebrates and fungi, improving ecosystem diversity and richness. This, in turn, can facilitate the nutrient cycle and dung degradation process, yielding benefits to soil quality, further supporting pasture productivity.

6.1.2 Veterinary intervention

Veterinary recommendations for TST were, in Chapter 3, supported by research showing how the technique can not only be beneficial to animal health but to dung fauna and the ecosystem services that they support. The implementation of TST could be used to yield benefits to animal health by providing refugia for anthelmintic-susceptible parasites (Cooke et al., 2017; Kenyon et al., 2009). This could reduce the development of anthelmintic resistance within the system, improving the long-term efficacy and sustainable use of anthelmintics as and when they are necessary (Charlier et al., 2014). Such a TST program could be effectively implemented through the implementation of general health checks, similar to the "Five Point Check" (Bath and van Wyk, 2009), with the inclusion of FECs. Information from these checks could then be used to select animals which need treatment or to undergo further, more specific, diagnostics to assess pathology. TST would also inhibit the insecticidal impact of anthelmintics on dung fauna, therefore facilitating invertebrate activity. The consequence of this might be an increase in dung degradation rate and nutrient turnover, in turn, improving soil fertility and organic matter content (Barth et al., 1993, 1994a). In addition, improved invertebrate activity may lead to the biological control of gastrointestinal nematodes, which can be consumed or buried by dung fauna (Bryan, 1976; Fincher, 1973).

This 'hypothetical farm' system is by no means claiming to be a perfect or superior system. Its purpose is to highlight how it is possible to better utilise biological mechanisms within agricultural systems, as opposed to the over-reliance on 'quick-fix' external inputs, in the form of fertilisers and anthelmintics, which has become endemic across the industry. Agricultural systems host an incredible diversity of biology, in the form of microbes, invertebrates, plants, fungi, and much more. We only understand a fraction of the capabilities of these organisms and even those alone are immense. Therefore, expanding this diversity has the potential to yield system-wide benefits far in excess of what one could predict.

6.1.3 Grazing rotation

Rotational grazing can potentially enhance pasture productivity whilst simultaneously controlling GINs and providing refugia for anthelmintic susceptible genes. A rotation with rapid cattle movement, but a long return time, such as mob-grazing can significantly increase pasture productivity (Campbell, 1969). Parasite control occurs through reducing exposure of

cattle to infective L3 larvae, by moving cattle onto new pasture before parasites are at peak infectivity (Barger et al., 1994; Marley et al., 2007; Stromberg and Averbeck, 1999). This was reflected by the mob-grazing case study which found low parasite burdens across all sites. The rate of parasite development can vary seasonally due to climatic variation (Hsu and Levine, 1977; Smith, 1990; Veglia, 1916). Therefore, a five day grazing period might be suitable in May, but be highly risky during June. This problem could be addressed by changing rotations in relation to climate, or by applying shorter grazing periods all year around. The prior of these options is most complex but could be most beneficial if achieved, whereas the second is simplest yet may not be as efficient in parasite control. Monitoring of GIN larval risk to cattle is possible, although resource intensive, and instead can be modelled to provide individual farm forecasts. The potential benefits of such a rotational grazing strategy are particularly pertinent to organic beef systems, however, are still highly relevant to non-organic farming, which could use external fertiliser inputs to further support pasture productivity if needed.

6.2 Future research

Whilst our understanding of beef production systems has been rapidly growing there are still numerous areas where research could yield significant benefits. It is essential to address these gaps in our knowledge in order to advance the industry so that it is resilient to current and future challenges. This is a significant challenge which requires multi-disciplinary scientific investigation and, most importantly, the drawing together of seemingly disparate lines of evidence. It is important to understand that no factor sits in isolation and that they are part of a complex and dynamic network. For example, improvements in soil quality, herbage composition, and biodiversity all influence one another. Three key areas for advancement are described below.

6.2.1 Dung composition

Dung is fundamental to nutrient recycling within beef systems, acting as a natural fertiliser. Whilst dung is highly valued in this regard; there is very little focus placed upon the exact composition and nutrient value of that dung. A factor which is driven by farm management decisions such as pasture composition and dietary supplementation. In contrast, the composition of artificial fertilisers is very precise. Whilst managing a farm to change the

nutritional composition of dung would be highly complex and impractical, it is important that we understand these differences. This is especially true for scientific studies that investigate nutrient dynamics with the goal of improving farm efficiency and sustainability. A study that takes great care to consider external inputs and system outputs would be fundamentally flawed if it were to not appropriately assess the characteristics of dung within that system. Chapter 2 highlights the significant differences that exist between dungs from different pastures, the extent of which is potentially far less than would have been seen if examining more diverse systems. Chapter 2 also describes a number of adapted and novel methodologies which can be used for the assessment of cattle dung in order to facilitate this advancement.

6.2.2 Disease and health assessment

A key area for advancement is the assessment of animal health and disease. Whilst the scientific capabilities exist, the application of these capabilities in a practical manner is lagging far behind what is seen in human medicine. Whilst this is somewhat to be expected, it still represents a clear area in which improvements can be made. This is particularly true for the diagnosis of GINs and other endoparasitic diseases. Whilst FECs are a highly practical and useful tool, they do not give the whole picture, reducing the effectiveness and sustainability of treatment programs. Chapter 4 provides a novel example of how faecal samples can provide significant information on animal health and immunology. Evidence from human medicine supports the utilisation of faeces as a diagnostic tool and tools such as CCA tests are examples of how these principles can be applied in a resource-efficient manner.

6.2.2.1 Mob-grazing

Chapter 5 provides scientific evidence supporting the reported benefits of mob-grazing. In particular, high levels of soil organic matter, high pasture productivity, and low burdens of GINs. This broad case study provides a platform to justify and inform future research into the practice, which could be a useful strategy for many farms, particularly organic ones. To truly assess this system a number of diverse and multi-disciplinary experiments must be conducted. This includes gathering case study evidence, conducting controlled field studies, and utilising computer modelling capabilities. A more thorough understanding of the implications of mob-grazing would allow for the more effective implementation of the

technique and give an understanding as to the situations in which it is appropriate and those in which it is not.

6.3 Concluding statement

The novel findings and outcomes of this study represent a progressive step towards further optimising pasture-fed beef production systems. Portions of the research are directly applicable to stakeholders at a farm level, whilst others outcomes yield a benefit to the scientific study of these systems. Further research into these topics and others within the field have the potential to advance the way in which we understand and manage livestock systems. The consequence of deepening our understanding is more efficient systems in terms of performance, profitability, and sustainability. This is not just desirable, but essential for livestock systems to be able to effectively respond to the challenges which they face and will continue to face in a changing world.

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Appendices

8.1 Weather data over degradation experiment

An on-site Met Office station recorded mean humidity (%), total rainfall (mm) and mean temperature each day (Figure 8.1). These factors are likely to have been one of the drivers of degradation through abiotic mechanisms such as physical breakdown by rain droplets, or by influencing biotic mechanisms such as aiding or inhibiting invertebrate activity. Nevertheless, these factors were consistent across the three field plots.

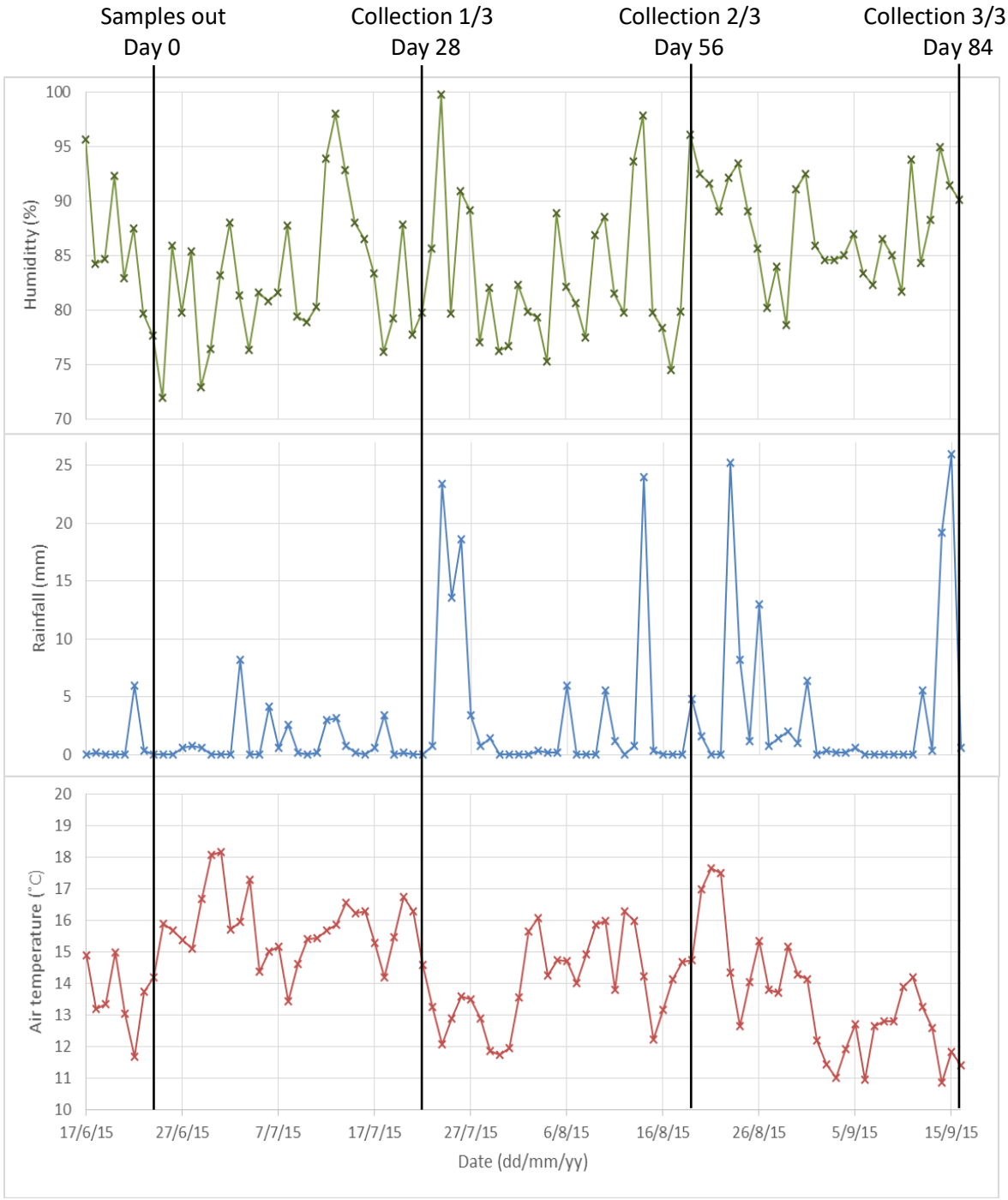

Figure 8.1 - Mean humidity (%), total rainfall (mm) and mean air temperature (˚C) of Rothamsted Research's North Wyke Farm Platform for each day of the study period and 7 days prior. Data gathered from an on-site Met Office station.

8.2 Randomised block design for degradation experiment

Randomised block design for the dung degradation experiment described in Chapter 2 (Figure 8.2).

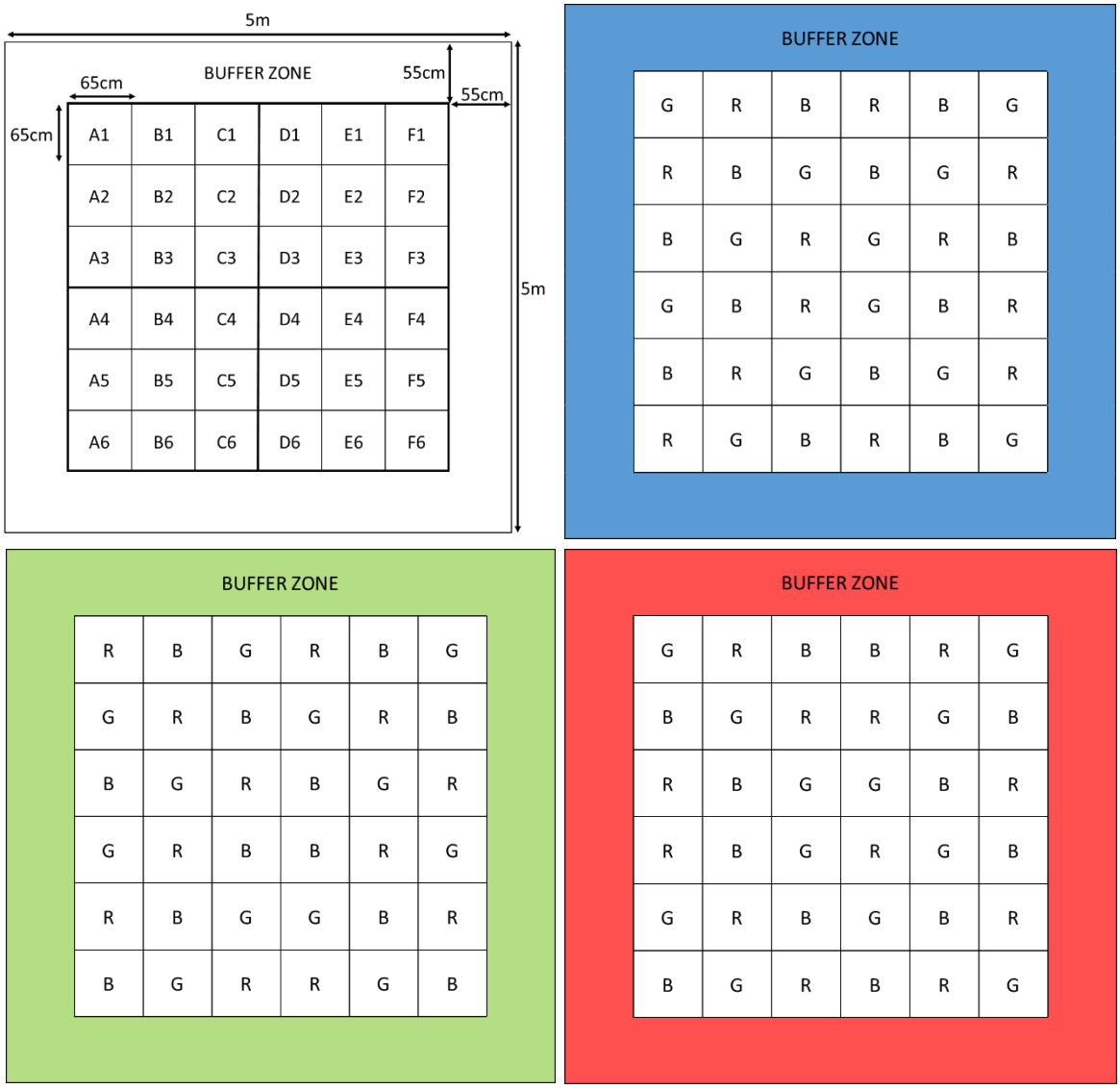

Figure 8.2 - Field plot designs. Clockwise from top left: Dimensions, grid references, and format of field plots. Field plot on the blue farmlet. Field plot on the green farmlet. Field plots on the red farmlet. Letters B, G, and R represent dung types blue, green, and red respectively. Not to scale.

8.3 Topographic map of field plots

Topgraphic map of field plots for the dung degradation experiment in Chapter 2 (Figure 8.3)

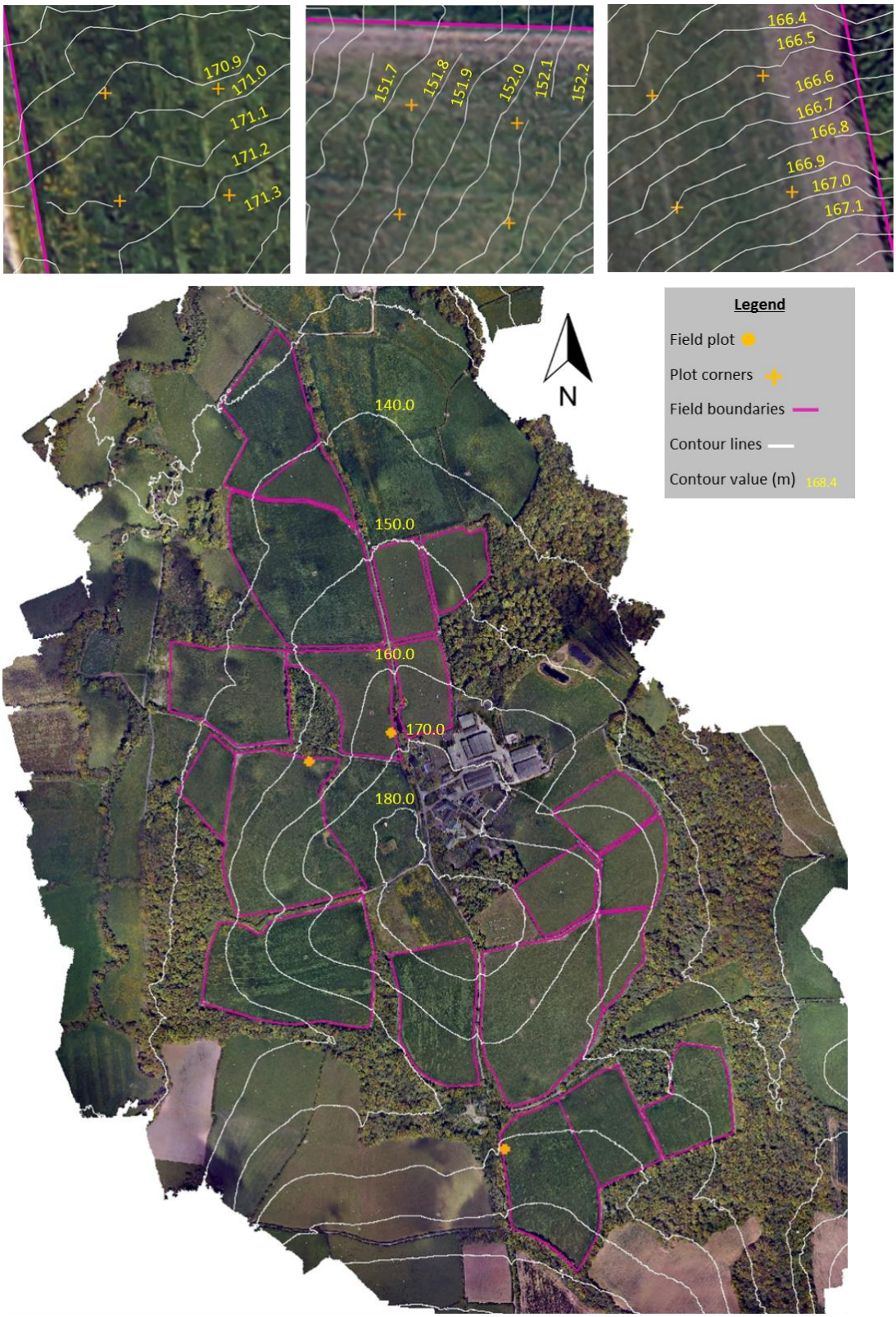

Figure 8.3 - Top: A map of each field plot showing 0.1m contours and plot corners. Below: Farm platform map including contour lines, and values, field boundaries, and location of field plots.

8.4 Full data of dung degradation

Table 2 - Full data for all measured biochemical components of all dung pats on the degradation of cattle dung on pasture. OM = organic matter, CLi = crude lipids, NDF = neutral detergent fibre, ADF = acid detergent fibre, ADL = acid detergent lignin, CP = crude protein/

Dung location (farmlet)	Dung source (farmlet)	Days on pasture	Nutrient values (%, dry matter basis)						
			Ash	OM	Cli	NDF	ADF	ADL	CP
n/a	Blue	0	21.0	79.0	4.9	47.4	35.6	16.7	17.9
n/a	Blue	0	21.0	79.0	7.2	46.9	35.3	16.9	17.4
n/a	Blue	0	22.4	77.6	3.6	46.1	37.1	18.1	17.5
n/a	Green	0	21.0	79.0	7.1	42.0	30.8	10.0	17.1
n/a	Green	0	21.0	79.0	6.6	44.8	32.2	16.4	17.1
n/a	Green	0	21.2	78.8	6.9	42.7	31.6	14.2	17.3
n/a	Red	0	19.1	80.9	7.4	43.7	32.0	10.3	19.3
n/a	Red	0	19.0	81.0	7.2	42.2	32.0	12.0	18.8
n/a	Red	0	18.4	81.6	7.4	42.1	32.5	11.8	18.7
Blue	Blue	28	23.1	76.9	2.0	48.5	39.9	16.5	16.7
Blue	Blue	28	27.5	72.5	3.3	48.0	40.1	17.3	16.6
Blue	Blue	28	22.5	77.5	2.7	49.4	37.7	18.7	17.4
Blue	Blue	28	23.4	76.6	2.9	56.1	39.1	16.9	17.7
Blue	Green	28	25.2	74.8	1.6	48.5	37.2	10.8	14.7
Blue	Green	28	24.5	75.5	2.5	81.0	38.2	12.4	16.4
Blue	Green	28	24.2	75.8	1.7	51.6	39.4	13.1	15.9
Blue	Green	28	27.0	73.0	3.8	48.2	38.4	12.0	15.4
Blue	Red	28	23.6	76.4	4.1	50.4	39.4	14.0	17.3
Blue	Red	28	23.3	76.7	3.0	51.6	40.1	16.3	16.9
Blue	Red	28	22.3	77.7	3.7	51.1	40.1	25.6	18.2
Blue	Red	28	22.6	77.4	4.0	54.6	38.7	15.3	17.1
Green	Blue	28	24.9	75.1	2.1	57.2	39.2	18.7	16.7
Green	Blue	28	25.0	75.0	2.6	49.2	39.7	17.4	17.1
Green	Blue	28	23.8	76.2	2.8	48.0	37.5	18.6	17.2
Green	Blue	28	24.8	75.2	1.7	48.4	40.0	18.3	16.4
Green	Green	28	23.0	77.0	3.6	49.3	36.9	18.0	17.0
Green	Green	28	22.9	77.1	3.8	29.1	36.4	18.5	18.5
Green	Green	28	23.9	76.1	2.6	54.6	37.2	12.0	17.1
Green	Green	28	25.8	74.2	3.4	49.8	34.8	14.0	17.6
Green	Red	28	41.1	58.9	2.3	46.4	37.7	16.8	18.9
Green	Red	28	21.0	79.0	3.2	46.7	36.9	14.1	18.6
Green	Red	28	22.9	77.1	2.5	52.5	38.2	16.9	17.5

Green	Red	28	22.3	77.7	3.0	49.7	36.2	14.0	18.9
Red	Blue	28	22.8	77.2	2.8	49.2	40.4	17.2	17.9
Red	Blue	28	24.2	75.8	2.2	49.0	41.6	18.8	16.6
Red	Blue	28	28.0	72.0	1.6	48.1	39.7	19.9	17.1
Red	Blue	28	24.6	75.4	2.0	46.2	40.2	19.5	18.3
Red	Green	28	23.3	76.7	2.8	58.9	37.3	16.0	18.2
Red	Green	28	23.6	76.4	2.6	51.1	36.4	12.1	16.7
Red	Green	28	23.4	76.6	2.9	49.2	53.6	15.1	17.3
Red	Green	28	24.5	75.5	3.4	51.5	37.6	12.9	16.8
Red	Red	28	21.3	78.7	4.4	48.4	37.5	13.3	18.5
Red	Red	28	22.0	78.0	2.8	49.3	38.2	14.1	18.0
Red	Red	28	20.6	79.4	3.1	50.2	40.5	15.2	18.0
Red	Red	28	26.9	73.1	2.6	51.1	37.8	12.6	16.9
Blue	Blue	56	25.2	74.8	2.6	62.4	41.9	19.6	18.7
Blue	Blue	56	26.7	73.3	2.7	49.9	39.4	21.2	18.0
Blue	Blue	56	26.0	74.0	2.4	50.3	38.5	10.1	18.0
Blue	Blue	56	33.6	66.4	3.2	54.5	44.7	21.7	17.0
Blue	Green	56	37.6	62.4	3.8	59.6	45.6	11.7	15.9
Blue	Green	56	44.0	56.0	4.1	50.8	49.9	9.5	13.6
Blue	Green	56	32.3	67.7	2.6	51.6	42.7	12.8	16.4
Blue	Green	56	30.3	69.7	2.2	48.1	42.3	13.1	17.0
Blue	Red	56	32.5	67.5	2.7	57.1	42.6	14.0	16.6
Blue	Red	56	Sample completely degraded before collection time						
Blue	Red	56	51.0	49.0	2.9	64.6		15.4	13.6
Blue	Red	56	21.7	78.3	2.6	48.2	36.2	13.8	20.3
Green	Blue	56	23.3	76.7	2.9	56.3	37.6	14.8	17.5
Green	Blue	56	25.4	74.6	2.5	54.9	38.1	18.9	19.1
Green	Blue	56	21.7	78.3	1.9	48.4	40.2	20.0	20.3
Green	Blue	56	24.9	75.1	2.5	52.0	39.7	17.6	18.5
Green	Green	56	26.5	73.5	2.8	53.9	40.7	21.0	18.0
Green	Green	56	26.2	73.8	3.9	50.8	40.8	14.1	15.8
Green	Green	56	26.1	73.9	3.2	48.8	37.1	11.7	18.6
Green	Green	56	11.0	89.0	2.1	49.4	37.4	13.9	19.1
Green	Red	56	25.7	74.3	3.4	58.7	41.8	17.6	17.7
Green	Red	56	20.5	79.5	2.3	49.1	36.7	16.0	19.7
Green	Red	56	23.4	76.6	1.5	49.0	42.2	18.4	19.2
Green	Red	56	22.3	77.7	1.6	48.0	40.6	17.7	18.7
Red	Blue	56	30.1	69.9	1.9	47.1	41.3	17.6	17.0
Red	Blue	56	31.8	68.2	2.0	59.2	44.7	17.7	17.1
Red	Blue	56	29.8	70.2	1.0	51.6	43.0	18.5	18.3
Red	Blue	56	35.1	64.9	1.2	54.3	46.2	17.4	16.4
Red	Green	56	49.1	50.9	2.6	67.8	53.0	10.7	13.0
Red	Green	56	23.3	76.7	1.5	57.5	36.1	12.4	18.3
Red	Green	56	31.9	68.1	1.7	55.5	45.7	15.9	16.5

Red	Green	56	36.7	63.3	1.8	52.6	47.4	13.1	15.3
Red	Red	56	23.0	77.0	1.9	53.3	40.1	15.2	19.5
Red	Red	56	28.7	71.3	2.7	61.2	44.8	21.6	18.8
Red	Red	56	44.9	55.1	2.1	54.9	56.2	13.6	13.8
Red	Red	56	26.5	73.5	3.3	48.1	39.3	13.2	19.7
Blue	Blue	84	30.9	69.1	1.4	53.1	45.5	19.9	20.0
Blue	Blue	84	32.3	67.7	2.0	49.2	44.4	17.2	18.5
Blue	Blue	84	31.0	69.0	1.9	49.0	44.1	18.0	18.0
Blue	Blue	84	74.1	25.9	0.0	72.0	71.3	8.1	6.3
Blue	Green	84	45.4	54.6	1.8	59.5	53.7	18.1	14.1
Blue	Green	84	41.6	58.4	2.3	56.0	51.8	12.3	13.5
Blue	Green	84	61.8	38.2	2.6	62.3	64.1	10.7	10.1
Blue	Green	84	67.5	32.5	1.7	66.5	67.5	9.2	7.4
Blue	Red	84	52.4	47.6	2.0	48.5	59.2	12.6	12.7
Blue	Red	84	62.0	38.0	2.9	79.1	46.3	13.8	13.9
Blue	Red	84	58.9	41.1	2.0	62.3	64.8	10.9	9.7
Blue	Red	84	70.9	29.1	0.2	67.9	70.4	9.1	8.2
Green	Blue	84	47.7	52.3	1.5	57.7	56.0	14.1	13.5
Green	Blue	84	47.1	52.9	3.1	55.2	55.9	17.8	15.0
Green	Blue	84	27.8	72.2	2.2	55.3	0.0	0.0	18.7
Green	Blue	84	47.2	52.8	1.3	52.0	56.9	14.0	13.6
Green	Green	84	42.8	57.2	2.3	51.7	51.7	11.9	14.5
Green	Green	84	26.7	73.3	2.6	42.3	39.6	15.6	18.7
Green	Green	84	56.4	43.6	1.9	74.5	34.6	14.0	11.1
Green	Green	84	36.6	63.4	0.3	52.2	48.0	14.6	15.9
Green	Red	84	37.1	62.9	1.6	55.1	48.6	16.6	18.4
Green	Red	84	61.2	38.8	0.6	49.1	65.9	11.1	10.8
Green	Red	84	50.4	49.6	2.8	62.7	56.6	10.8	13.8
Green	Red	84	23.0	77.0	2.8	49.7	32.1	16.1	19.7
Red	Blue	84	54.7	45.3	1.2	51.3	64.8	11.8	10.9
Red	Blue	84	56.0	44.0	1.1	73.4	60.4	14.3	13.8
Red	Blue	84	82.9	17.1	0.5	56.5	79.9	6.7	4.7
Red	Blue	84	72.6	27.4	0.0	89.5	71.9	9.2	6.8
Red	Green	84	83.9	16.1	1.0	66.5	79.6	6.6	4.4
Red	Green	84	64.0	36.0	1.5	56.7	66.7	14.3	8.8
Red	Green	84	70.0	30.0	0.0	56.9	70.4	10.0	8.3
Red	Green	84	78.5	21.5	1.3	49.4	77.2	7.4	6.8
Red	Red	84	70.9	29.1	0.7	54.2	71.5	9.0	8.0
Red	Red	84	68.8	31.2	1.3	57.9	71.8	11.3	9.3
Red	Red	84	76.3	23.7	0.9	86.7	74.8	6.9	6.2
Red	Red	84	77.7	22.3	1.2	73.8	78.7	7.6	6.1

8.5 Error table for micronutrient analysis

XRF analysis yielded a wide range of errors (ppm) between the different elements quantified (Table 8.3),

Table 8.3 - Error of micronutrient concentrations (ppm).

	Error of concentration of element in material (ppm)											
	Feed						Dung					
	Silage			Herbage			Silage diet			Herbage diet		
	B	G	R	B	G	R	B	G	R	B	G	R
Al	310	325	344	311	321	347	351	357	347	304	309	345
As	1	1	1	1	1	1	1	1	1	1	1	1
Br	1	1	1	1	1	1	1	1	1	1	1	1
Ca	674	607	1104	686	991	779	1230	812	1210	1265	811	1125
Co	1	1	1	1	1	1	2	1	2	1	1	2
Cr	22	18	35	20	27	18	23	21	31	25	15	29
Cu	13	11	36	13	24	16	34	12	27	23	11	22
Fe	29	26	76	42	38	24	76	72	93	62	41	93
K	1903	1711	3311	1581	2564	2183	1216	1265	2231	1881	984	1762
Mg	999	1035	998	963	1048	1061	1203	1151	1136	1054	1106	1046
Mn	28	24	41	26	33	25	42	34	54	57	34	50
Na	13148	13376	12808	13254	13009	13754	13778	13040	12793	12945	13430	12916
Ni	0	0	0	0	0	0	0	0	0	0	0	0
P	60	67	63	63	68	74	103	97	94	87	107	86
S	41	47	45	47	48	54	54	52	53	48	55	49
Se	1	1	1	1	1	1	1	1	1	1	1	1
Zn	11	10	19	11	16	12	18	13	18	19	14	18

8.6 Comparison of Kjeldahl and Dumas methods

8.6.1 Introduction

Crude protein cannot easily be quantified directly, and therefore it is standard practice for nitrogen to be used as a proxy. Quantified nitrogen is multiplied by 6.25 in order to provide an estimation of crude protein. There is significant debate in how nitrogen, for crude protein analysis, should be quantified - the historical Kjeldahl method, or the more modern Dumas technique. The Kjeldahl method is often favoured due to its long-standing use in forage science; it is also the technique that the nitrogen-protein conversion factor was based on. However, the technique is incredibly resource costly, requiring a wide array of reagents and multiple steps (Bellomonte et al., 1986). With each step in the process the potential errors

compound, therefore results may not be consistently reliable. In contrast, the Dumas technique is much simpler and quicker, samples undergo flash combustion at approximately 900°C, and an automated process quantifies nitrogen based on thermal conductivity. Results from the two methods are generally comparable (Bellomonte et al., 1986; Simonne et al., 1997; Wiles et al., 1997), with Dumas often yielding marginally higher nitrogen results (Thompson et al., 2002; Wiles et al., 1997). Studies typically focus on foodstuffs with little work conducted on dung. However, findings by Stitcher et al. (1969), on dung samples, were concurrent with work on foodstuffs. While these are the two most common methods used for CP quantification, other techniques are available, such as NIRS (near infrared spectroscopy) which works by measuring the absorption of near infrared radiation and applying results to a model which associates absorption with different compounds (Barton and Windham, 1988).

8.6.2 Method

In order to determine which method of crude protein quantification, Kjeldahl or Dumas, was to be used, a comparison was conducted. Twenty-two of the dung samples collected from the degradation analyses underwent protein determination by both Kjeldahl and Dumas techniques.

Quantification by Dumas was conducted as per above (2.2.3.6 Crude protein). The procedure for Kjeldahl analysis was as follows. 0.5g of sample was weighed into digestion tubes along with two Kjeldahl catalyst tablets (Kjeltab CK) and 15ml of 98% sulphuric acid. Digestion tubes were added to a centrifugal scrubber unit (Gerhardt TURBOSOG) to remove acid fumes. The scrubber was prepared with 1L of 20% sodium hydroxide. Digestion tubes were fitted into the scrubber unit, with spare spaces filled with empty digestion tubes. The exhaust head was then fitted to the racked tubes, and the scrubber was then initiated. Simultaneously the distillation unit (Gerhardt Vapodest 40) was purged to remove trapped air and older chemical residues. The unit was connected to reservoirs of 40% sodium hydroxide, 4% boric acid, and deionised water. The connected tubes were then rinsed through with their relevant liquid. The digestion tubes were then removed from the scrubber unit and, one by one fitted to the distillation unit along with a clean 250ml receiving beaker. During distillation the released nitrogen compounds and incorporated into the boric acid receiving solution, thus changing its pH.

Nitrogen was then determined by titration (Equation 8.1) and converted to protein using a conversion factor of 6.25.

$$\%N = (titrant\ volume\ (ml)\ x\ acid\ normality\ x\ 1.40067) \div sample\ weight\ (g)$$

Equation 8.1 - Titration equation for the Kjeldahl protocol.

A paired *t*-test and a Pearson's correlation was conducted on the data to determine the presence of significant differences in the results gained from each method and to determine if the results correlate significantly.

8.6.3 Results

The paired *t*-test between nitrogen values determined by the Kjeldahl method and determined by Dumas method showed a statistically significant difference between results (n = 22, $t = 3.34$, $p = 0.003$). The Dumas data set had a higher mean value of 2.585, compared to 2.385 by Kjeldahl and also had a lower standard deviation of 0.538 compared to Kjeldahl's 0.638. The Pearson's correlation (Figure 8.4) between the two data sets was 0.900 ($p < 0.0005$), which is low when considering that both methods aim to quantify the same compound. This trend is concurrent with trends in the literature, however the majority of the literature compares Kjeldahl and Dumas for foodstuffs and animal feed and there is a notable variation in results of comparisons between different sample types (Jung et al., 2003; Miller et al., 2007; Simonne et al., 1997). Results support Hypothesis 5.

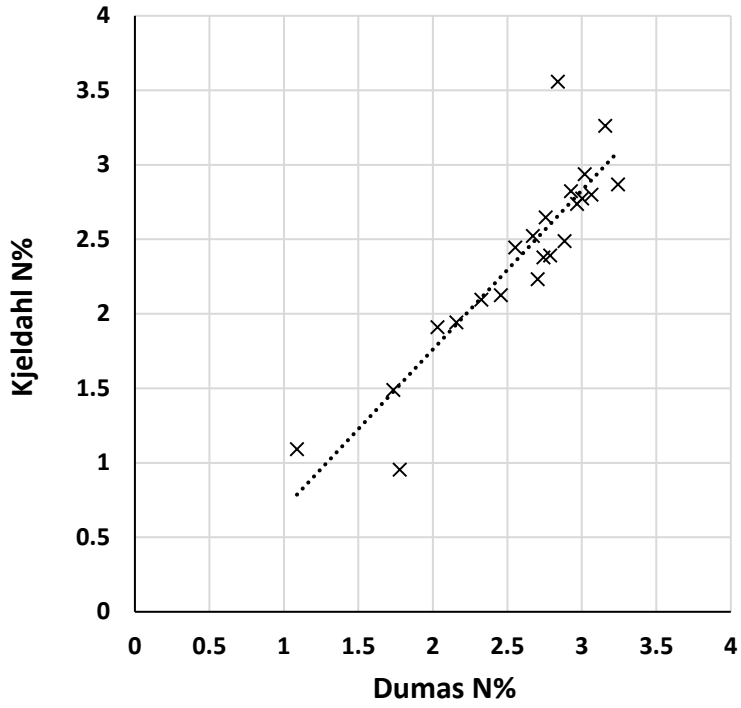

Figure 8.4 - Scatterplot of N content of samples for both Kjeldahl and Dumas methods (n = 22).

8.6.4 Discussion

Results favoured the Dumas method, as opposed to Kjeldahl, for the quantification of protein in agricultural samples, particularly of dung, which had received little attention in the past. Results between the two methods correlated significantly, however, were typically slightly higher for Dumas. At this stage, there is no saying which is more accurate, and there may be a need to adjust the conversion factor in the future if Dumas overestimates. Overall Dumas was far cheaper, quicker, and safer than Kjeldahl. In concurrence with the literature, it is recommended that Dumas should be used as the standard method for protein quantification (Bellomonte et al., 1986; Jung et al., 2003; Miller et al., 2007; Simonne et al., 1997; Stitcher et al., 1969; Thompson et al., 2002; Wiles et al., 1997). The use of Kjeldahl for historical purposes is not justifiable given the low efficiency of the technique and the increasing body of evidence favouring Dumas combustion.

8.7 Cold carcass weights

Cattle from the green farmlet had both highest cold carcass sale price (£GBP) at slaughter and the highest price paid per kilo of carcass weight (a factor based upon conformity and carcass

fat content), making them the most financially valuable animals, although the difference was not significant, as determined by a one-way ANOVA ($F = 1.43$, $p = 0.246$) (Figure 8.5).

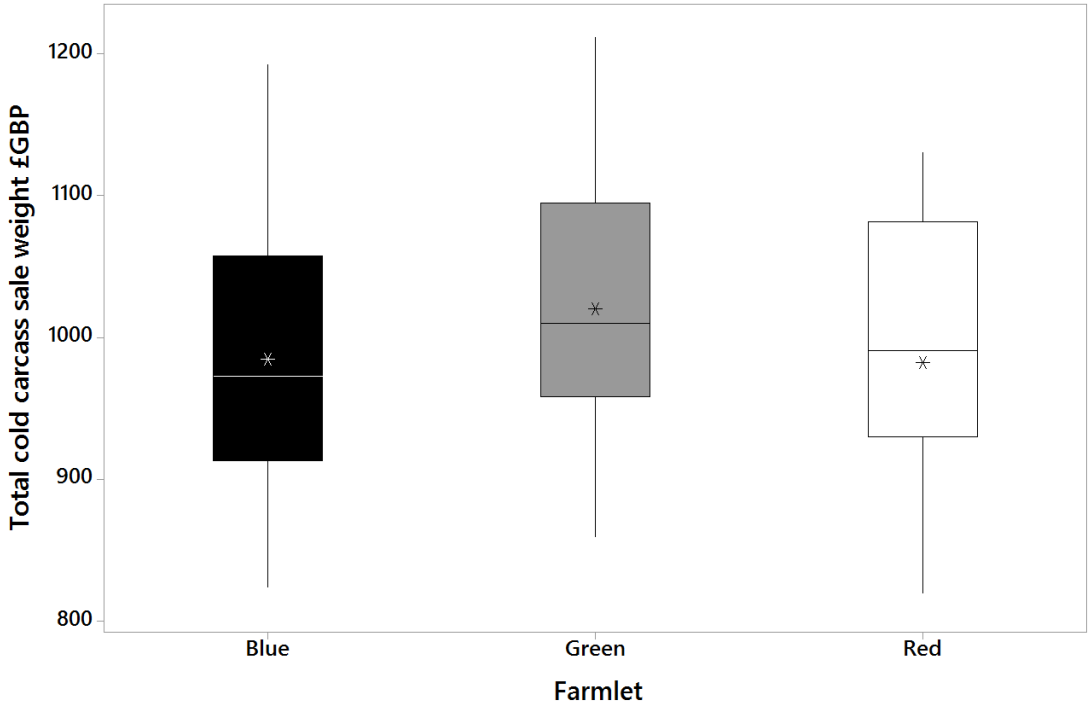

Figure 8.5 – Boxplots of total cold carcass sale price (£GBP) of cattle, from the three different farmlets, at slaughter (Winter 2015/16). Asterisks represent mean.

8.8 Modelling paper

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Modelling the impact of targeted anthelmintic treatment of cattle on dung fauna

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ABSTRACT

The insecticidal properties of many anthelmintics pose a risk to dung fauna through the effects of drug residues in dung on the activity, oviposition and development of dung-dwelling invertebrates. Reductions in dung fauna numbers can inhibit dung degradation, which may impact biodiversity and nutrient cycling on farms. A simulation model was created to predict the impact of antiparasitic drugs on cattle dung fauna, and calibrated using published data on the dung-breeding fly *Scathophaga stercoraria*. This model was then tested under different effective dung drug concentrations (EC) and proportions of treated cattle (PT) to determine the impact under different application regimens. EC accounted for 12.9% of the observed variation in *S. stercoraria* population size, whilst PT accounted for 54.9%. The model outputs indicate that the tendency within veterinary medicine for targeted selective treatments (TST), in order to attenuate selection for drug resistance in parasite populations, will decrease the negative impacts of treatments on dung fauna populations by providing population refugia. This provides novel evidence for the benefits of TST regimens on local food webs, relative to whole-herd treatments. The model outputs were used to create a risk graph for stakeholders to use to estimate risk of anthelminthic toxicity to dung fauna.

1. Introduction

Anthelmintic drugs are widely and routinely administered to grazing livestock to control gastrointestinal nematodes and other parasites. Anthelmintics are typically not fully metabolized within the host animal and residues of the drugs are often excreted in dung (McKellar et al., 1993) (and urine (McKellar, 1997)) and can therefore exert non-target effects on invertebrate fauna which spend part, or all, of their life cycle in dung (Floate, 1998a, 1998b; Gover and Strong, 1995; Madsen et al., 1990; Sommer et al., 1992; Sutton et al., 2014) and also on soil invertebrates (Scheffczyk et al., 2016). Such effects include inhibited motility, oviposition, emergence, and reduced dung pat colonisation (Floate, 1998a, 1998b; Gover and Strong, 1995; Suarez et al., 2003). Invertebrate dung fauna significantly contribute to the degradation of dung through physical processes and therefore reductions in the activity and populations of degradative fauna can to slow dung degradation (Madsen et al., 1990; Wall and Strong, 1987) with potential knock-on effects on important local processes., including local ecology (Beynon, 2012; Strong, 1993; et al., 2012; Wall and Beynon, 2012) and epidemiology. In recent years, the mounting resistance of gastrointestinal parasites of domestic livestock to anthelmintic drugs has led to a shift away from whole-herd treatments, and recommendations for targeted selected treatment (TST) (Charlier et al., 2014) of only part of the herd. This strategy aims to generate refugia from drug exposure among parasite populations, slowing the development of resistance. In principle, refugia from drug residues ought also to be generated for dung fauna, supporting their populations; however, to date no systematic attempts have been made to evaluate this possibility.

The ability to assess and predict the impact of anthelmintics and other routine veterinary medicines on the wider environment is essential for informed drug development and policy in agriculture. In particular, parasite control practices that slow the development of resistance to commonly administered anthelmintics are essential to sustainable livestock production systems. However, the scale and complexity of the drug-dung-fauna system is challenging to observe and quantify *in vivo* and is difficult to fully represent under controlled laboratory conditions. Modelling techniques are the best alternatives to address these issues by allowing for the manipulation of a wide range of variables specific to individual field scenarios, and rapid assessments of the potential impacts of new parasite control and other management

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practices on dung fauna. Boxall et al. (2007) developed a screening index for assessing the impact of veterinary medicines on dung flies. The index was simple and allowed for estimates to be calculated with relatively small amounts of data, allowing for rapid screening of multiple drugs. The index assessed impact by multiplying three variables: proportion of cattle treated, proportion of time of faunal contact with dung, and dung toxicity. A central assumption was that the three variables are equally weighted, but this assumption inadvertently creates a potential mathematical ceiling to drug toxicity. Vale and Grant (2002) took a different approach in their development of a model to assess the impact of insecticide-contaminated dung on dung fauna. The model considered a broad and novel range of variables including the response to distinct adverse ecological events on insect life cycle stages and dung-insect interactions which aided the understanding of the importance of refugia for the ecology different species of invertebrates.

Here, we test the hypothesis that the proportion of cattle treated (PT) with anthelmintics has a greater influence on *Scathophaga stercoraria* populations than the strength of drug residue in dung (EC). We build on previous theoretical and modelling approaches to create a new modelling approach to simulate the drug-dung-fauna system and evaluate the potential impacts of antiparasitic drug use in grazed cattle production systems. We use the model to consider how varying treatment regimens administered by veterinarians for the purpose of livestock health and welfare have non-target influences on dung invertebrates, and to provide a risk graph to inform stakeholders in sustainable livestock production systems.

2. Methods

2.1. Model description

A simulation model was created using NetLogo 5.0.4 (Wilensky, 1999) to estimate the impact of a hypothetical anthelmintic that expressed insecticidal properties when excreted in dung by cattle in a grazed field, upon a model dung invertebrate. A 2-dimensional virtual pasture system was created, occupied by a herd of cattle and a population of the model invertebrate. All actions and interactions presented were simulated hourly time-steps for each individual cattle or invertebrate, as appropriate.

2.2. Model components

The model simulated the interaction between a model dung invertebrate and cattle defecation behaviour, and the potential for invertebrate survival to be changed by different concentrations of anthelmintic residues in the dung.

The model invertebrate was the yellow dung fly *Scathophaga stercoraria*. The model utilized published data (Table 1) to simulate the life cycle of *S. stercoraria* in a temperate cattle grazing system. *Scathophaga stercoraria* is a well-studied dung fauna species, for which detailed information on life cycle parameters is widely available. The species is highly abundant across the northern hemisphere, and some of its life cycle stages are dependent on dung.

The model cattle were based on published data on temperate grazing commercial beef and dairy herds (Table 1). There were two components to cattle behaviour: (1) defaecation frequency, and (2) randomized movement across a field. The cattle were treated or untreated with a hypothetical anthelmintic, producing toxic or non-toxic dung, respectively. The proportion of cattle treated (PT) ranged from 0 to 1 in increments of 0.1 and was specific as an independent variable in each simulation,

The rate of defecation of model dung by the model cattle and its mean carrying capacity for *S. stercoraria* was based on published data for temperate commercial beef and dairy systems (Table 1). The model dung were toxic or non-toxic. The strength of the toxicity, i.e. effective concentration (EC) ranged from 0 to 1 in increments of 0.1 and was specific as an independent variable in each simulation. The dung became unattractive for *S. stercoraria* regardless of toxicity after a simulated 120 h.

Table 1
Model variables and values used for simulations. Mean values are fixed constants other than those with a standard deviation (S.D.) which were random variables within a standard normal distribution generated by random number generator using NetLogo 5.0.4. Sources: [1.] Blanckenhorn, (1997), [2.] Blanckenhorn et al. (2010), [3.] Römbke et al. (2009), [4.] Martin et al. (2004), [5.] Aland et al. (2002), [6.] Gary et al. (1970), [7.] Oudshoorn et al. (2008), [8.] Sahara et al. (1990), [9.] Villettaz Robichaud et al. (2011), [10.] Floate (1998), [11.] Vale and Grant (2002), [12.] Geiger (2010), [13.] Parker (1970).

Variable[source]	Value
Dung fauna (*S. stercoraria*)	
Adult life span (emergence to death)[1]	44 days
Juvenile period (egg to emergence)[2]	22 days
Female:male ratio[1]	1:1
Dung preference[3]	0
Progeny to reach adulthood[4]	10.8 (2.9)
Cattle and dung	
Mean daily defecation rate (pats per day)[5–9]	11.2 (2.4)
Dung attractive period (with drug residue) to *S. stercoraria*[3,10,11]	5 days
Dung attractive period (no drug residue) to *S. stercoraria*[3,10,11]	5 days
Mean dung pat carrying capacity for juveniles[12]	4.3
Season length[13]	6 months
Number of cattle	20

A starting population of 100 individuals of *S. stercoraria*, covering a random distribution of ages within typical life expectancy for *S. stercoraria*, were simultaneously introduced to the system. They actively sought out cattle dung in order to produce off-spring with no preference for toxic or non-toxic dung. Population fitness responses of the *S. stercoraria* to contact with toxic dung was based on the interaction between PT and the specific EC.

Primary assumptions were:

(i) the model dung toxicity retained a constant toxicity for 120 h
(ii) there were no sub-lethal effects of the anthelmintics upon *S. stercoraria*
(iii) there were no other sources of mortality exist for *S. stercoraria* other than toxicosis or exceedance of life span
(iv) the population of *S. stercoraria* is isolated.

No values or weightings of variables within the model were assumed or given arbitrary values.

2.3. Application of modeling approach

The model was run 605 times. Each run simulated 4380 h (6 months) using all combinations of 11 PT values and 11 EC values, totaling 121 unique sets of parameter values. There were five repeats of each set, with variable outcomes depending on values simulated from normal distributions: the mean of each set of repeats was used for statistical analyses. The Anderson-Darling normality test was conducted on residuals for the dependent variable of final population size at the end of the simulated period to ensure appropriateness for parametric testing. This was followed by Pearson's correlation analyses of final population size versus PT and EC. Multiple regression analyses were then conducted to attribute how much of the variation in final population size was due to PT and EC, respectively.

A number of individual paired simulations were run to evaluate the index created by Boxall et al. (2007). These simulations were performed in pairs in which the product of PT and EC were equal, but the individual values of PT and EC in each pair were not equal. To achieve this the values for PT and EC of pair 1 were switched to form pair 2 (Table 2). For the Boxall et al. (2007) model to agree with the presented model, there should be no significant different between pairs that meet the aforementioned assumptions. Final population numbers from

Table 2
Values of PT and EC for paired simulations in order to evaluate Boxall et al. (2007) model.

	Group A		Group B	
Pair no.	EC	PT	EC	PT
1	0.0	1.0	1.0	0.0
2	0.1	0.9	0.9	0.1
3	0.2	0.8	0.8	0.2
4	0.3	0.7	0.7	0.3
5	0.4	0.6	0.6	0.4

simulations were then subject to the Paired *T*-test.

3. Results

The distribution of final population sizes across all simulations was non-normal (Anderson-Darling, $p = < 0.005$). The data shows two distinct groupings based on final population size, one at 0 and the other in the region of 3100–4300 (Fig. 1). This latter group, the 'maximum fitness' group, had a normal distribution ($p = 0.383$). Quartiles for the maximum fitness group were measured as Q0 = 3259, Q1 = 3597, Q2 = 3703, Q3 = 3798, Q4 = 4197. PT and EC combinations that resulted in final populations of < Q0, and therefore outside of this group, were considered as high risk. Combinations that fell between Q0 and Q1 were considered medium risk, and all over combinations resulting in final populations > Q1 were considered low risk (Fig. 2).

In general, incremental increases in PT and EC at low levels had little effect on final population size (no. of individuals), but a tipping point was reached beyond which the population decreased exponentially (Table 3). Rising EC values from 0.0 to 0.5 brought about gradual decreases in final population size; however, as EC exceeded 0.5 its effect on population size reduced. In contrast, rising PT values of 0.0–0.5 had little impact upon population sizes, but as PT exceeded 0.5 there was a rapid drop in population size.

The residuals of the complete data for all experimental runs were normally distributed (as tested by Anderson-Darling test, $p = 0.281$) and thus no transformation was required for parametric analyses. A Pearson's correlation analysis showed that final population size was significantly correlated with PT (-0.694, $p = < 0.001$) and EC (-0.336, $p = < 0.001$). A subsequent multiple regression calculated the total variance of final population explained by PT and EC together, R^2, to be 67.8% ($p < 0.001$). Further individual regressions showed that PT explained 54.9% ($p = < 0.001$) and EC explained 12.9% ($p = < 0.001$) of total variance in final population size.

The paired *t*-tests, for the purpose of evaluating Boxall et al., showed a statistically significant intra-pair difference ($t = 2.43$, $p = 0.023$) and therefore the H_0 was rejected in favour of the H_a, that there is an intra-pair difference. That is: simulations of which the sum of PT and EC are equal do not yield equal results.

4. Discussion

In this study, we used a novel simulation to test the hypothesis that PT had a greater impact on the population size of *S. stercoraria* than EC. The outcomes of 605 simulations of 121 parings of PT and EC indeed indicate that this hypothesis can be accepted. The distribution of data predicted that populations of *S. stercoraria* were generally resilient and can maintain stable numbers up until a tipping point at which mortality becomes probable. As such, our model develops the concept of the screening level index (Boxall et al., 2007) through simulation modelling using published data about key life cycle parameters that could strongly influence drug-insect interactions. We propose that this new approach provides a better justified mechanistic framework for impact assessment, which will improve recommendations of use of veterinary medicines with consideration for livestock dung ecology and wider impacts on the environment.

Cow pats in grazed systems without drug residues may provide an important reservoir of biodiversity, allowing maintenance populations of coprophagic fauna that are important for ecosystem services including nutrient cycling, carbon cycling and soil quality, e.g. dung beetles and insect larvae. Therefore, TST, as opposed to a whole-herd treatment, is recommended to reduce the impacts of drug treatment on local ecosystems, with additional economic benefits through reduced inputs on-farm (Charlier et al., 2012), ecosystem service delivery, and ensuring sustainable parasite control options in the longer term through slowing of anthelmintic resistance. However, in our model, *S. stercoraria* populations were assumed to be isolated, but wider consideration of the spatial variation in the local food web including the availability of drug-free dung in the wider environment would more closely represent the complexity of farming systems.

Our model provides a framework that is adaptable to dung-breeding insect species other than *S. stercoraria*. Its application to other target

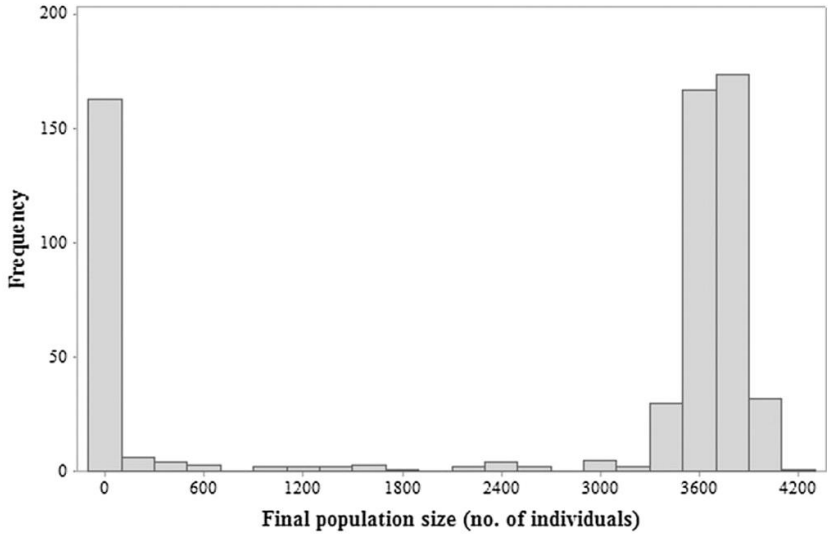

Fig. 1. Distribution of final population sizes of *S. stercoraria* from all (605) simulations of PT and EC pairings.

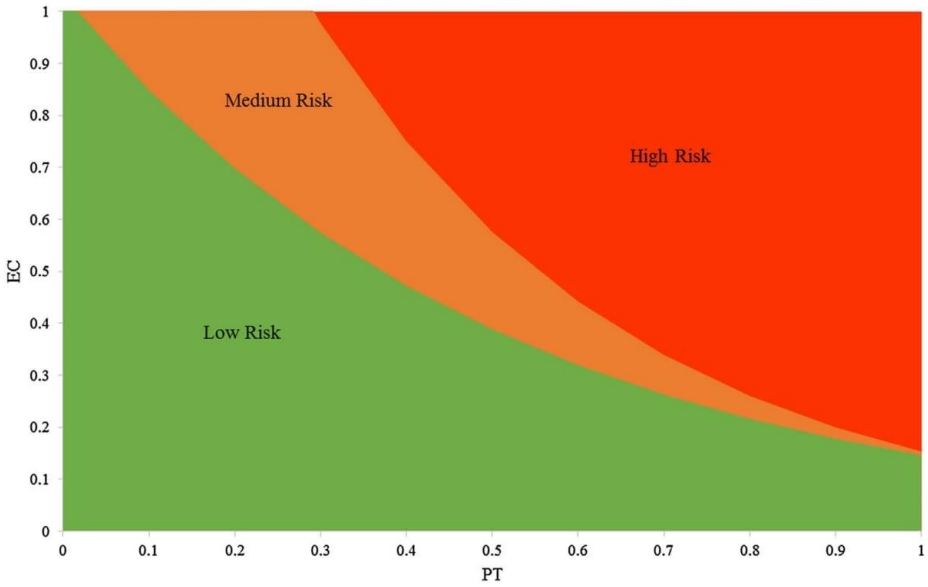

Fig. 2. Risk thresholds for the impact of anthelmintics on *S. stercoraria*. "Low Risk" refers to PT (proportion of treated cattle) and EC (effective concentration) combinations that result in final populations exceeding Q1 of the maximum fitness group, "Medium Risk" to those falling between Q0 and Q1 and "High Risk" to those falling below Q0.

species, however, would require further empirical information on the toxicity of various drugs, as faecal residues, on specific fauna. Moreover, life cycle parameters specific to other species would be required, although the model could also be used to explore parameter space and identify broad characteristics of species that are likely to be vulnerable to anthelmintic residues in dung, and the extent to which these might be attenuated by TST. Since the model framework was developed using a bottom-up approach, it lends itself to constructive adaptation and expansion. With sufficient observational data there is scope for future models, within such a framework, to increase in complexity and realism. Expansion of the model to represent multiple invertebrates at farm level would enable holistic landscape-scale impact assessments and attenuation strategies.

The use of veterinary medicines, with non-target insecticidal properties, is ubiquitous and therefore the applicability of observed results may be equally wide, and this model framework can be adapted to any system, anywhere, given workable parameter estimates. The data from the literature that provided the foundation for the model was predominantly derived from studies in temperate regions, and we recognize that climatic differences may have a significant impact on the ecotoxicity of such medicines (Kryger et al., 2005). Moreover, the model was based upon a set-stocked system but could be modified to represent more extensive systems, including ranch/range rearing of cattle in the USA, South America, and Australia. The model provides a framework for the development of future similar work and could be applied to scenario-testing using the specific characteristics of different cattle production systems across the world.

Despite the high profile, global threat of drug resistance, the long-term impacts of drugs, especially antiparasitics with non-target insecticidal properties, are largely unknown. The topic is a key area for future work to enable effective assessment and regulation of the use of veterinary medicines, with regards to their impact on all aspects of biodiversity (Adler et al., 2016). Future work should also include economic analysis, in order to balance short-term production gains with longer term environmental impacts. There is likely to be a utilitarian argument to use veterinary medicines in a more sustainable manner, including the utilization of preventative and non-pharmaceutical methods (Kaplan and Vidyashankar, 2012; Papadopoulos, 2008;

Table 3
Mean simulated final population size for varying proportions of cattle treated (PT) and effective dung drug concentrations (EC). PT and EC range from 0 to 1.0 in intervals of 0.1, so simulations were conducted for 121 scenarios, representing every PT and EC value combination.

		PT										
		0.0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1
EC	**0.0**	3678	3676	3701	3763	3645	3739	3770	3696	3590	3642	3608
	0.1	3675	3681	3734	3699	3652	3765	3767	3650	3574	3598	3713
	0.2	3764	3697	3692	3685	3659	3629	3741	3635	3044	1353	338
	0.3	3789	3753	3655	3777	3659	3777	3409	2950	1422	0	0
	0.4	3859	3721	3758	3722	3603	3789	2784	1756	0	0	0
	0.5	3667	3620	3705	3807	3294	3325	2112	0	3	0	0
	0.6	3658	3661	3701	3777	3783	3147	1481	85	0	0	0
	0.7	3738	3786	3816	3723	3755	2902	1385	762	0	0	0
	0.8	3750	3661	3671	3655	3660	3310	1634	744	4	0	0
	0.9	3761	3665	3689	3724	3178	2594	1586	8	0	0	0
	1	3790	3680	3754	3564	3745	2337	0	724	0	0	0

Wolstenholme et al., 2004). The emergence of part-herd anti-parasitic treatments, or TST, is an example of a more efficiently targeted approach to chemical utilization in agricultural systems, which has potential long-term economic benefits, as well as reduced environmental impacts. The current model shows this synergy in quantitative terms for a model insect species, and provides a framework for impact assessment and optimization of TST strategies across a wider range of dung fauna, including those of conservation relevance.

Author contributions

All authors contributed to drafts of the paper. EM devised the study and advised on model assumptions and parameter values. AC compiled and ran the model, analyzed results and led the writing of the paper. JD supervised AC and provided editorial support.

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8.9 Faecal antibody detection (FAD) paper

Parasitology

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Research Article

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The latest FAD – Faecal antibody detection in cattle. Protocol and results from three UK beef farms naturally infected with gastrointestinal nematodes

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Abstract

Antibodies at gastrointestinal mucosal membranes play a vital role in immunological protection against a range of pathogens, including helminths. Gastrointestinal health is central to efficient livestock production, and such infections cause significant losses. Fecal samples were taken from 114 cattle, across three beef farms, with matched blood samples taken from 22 of those animals. To achieve fecal antibody detection, a novel fecal supernatant was extracted. Fecal supernatant and serum samples were then analysed, using adapted enzyme-linked immunosorbent assay protocols, for levels of total immunoglobulin (Ig)A, IgG, IgM, and *Teladorsagia circumcincta*-specific IgA, IgG, IgM and IgE (in the absence of reagents for cattle-specific nematode species). Fecal nematode egg counts were conducted on all fecal samples. Assays performed successfully and showed that IgA was the predominant antibody in fecal samples, whereas IgG was predominant in serum. Total IgA in feces and serum correlated within individuals (0.581, $P = 0.005$), but other Ig types did not. Results support the hypothesis that the tested protocols are an effective method for the non-invasive assessment of cattle immunology. The method could be used as part of animal health assessments, although further work is required to interpret the relationship between results and levels of infection and immunity.

Introduction

Infection of cattle with parasites, especially gastrointestinal nematodes (GINs), incurs important economic losses, while options for control are undermined by anthelmintic drug resistance. Targeted selective treatment (TST), whereby drugs are provided only to individuals in greatest need, has the potential to yield long-term benefits to animal health at individual, herd and national levels due to its mitigating effect on the selection of drug-resistant pathogens (van Wyk *et al.*, 2006; Charlier *et al.*, 2014). Central to TST strategies is the need for comprehensive animal health assessments, used to select individuals for treatment (Bath and van Wyk, 2009; Bentounsi *et al.*, 2012; Charlier *et al.*, 2014). This screening process can involve a range of non-specific health indicators, such as weight gain, body condition and evidence of diarrhoea, in tandem with more specific indicators of infection such as fecal egg counts (FECs). A significant drawback of FEC techniques is that egg counts are not necessarily indicative of parasite burden, or of consequent pathology or impact on health. The advancement of TST requires the development of new, high throughput diagnostics that are able to assess physiological parameters of animal health, especially in relation to GINs. Fecal antibody detection (FAD) is a candidate to join this tool kit of techniques, allowing for more detailed and comprehensive evaluations of animal health, therefore enhancing current TST strategies.

Gastrointestinal health is particularly important for efficient feed conversion within livestock production systems and general animal health. The gut wall acts as an interactive barrier between the external environment and the rest of the animal's systems, allowing for the passage of beneficial nutrients into the body. However, the gut wall is also a primary entry point and barrier for ingested pathogens that have the potential to cause extensive physiological damage and ultimately reduce nutrient utility and subsequent animal health and performance (Sykes *et al.*, 1975; Smith *et al.*, 1985; Parkins and Holmes, 1989; Poppi *et al.*, 1990; Coop and Holmes, 1996; Claerebout and Vercruysse, 2000). A key component in this defence against pathogens is the immune system and its response at mucosal membranes (Miller, 1987; Nawa *et al.*, 1994; Onah and Nawa, 2000; Nagler-Anderson, 2001; Sansonetti, 2004) including immunoglobulins (Ig), which directly combat pathogens and other foreign bodies. Each of the five Ig isotypes (IgA, IgD, IgE, IgG and IgM) has numerous subtypes that play different roles and are generally localized to specific systems or tissues.

Heightened antibody levels are often symptomatic of disease challenge and an individual's response to that; however, challenge does not necessarily relate to pathology if an animal is

coping (Newkirk *et al.*, 2005; Dong *et al.*, 2008). Over the course of an infection, antibody levels vary greatly and are typically characterized by a primary and secondary response; therefore, antibody levels at a single time point may not necessarily correlate with disease burden. Antibody levels are widely measured to indicate exposure, response and tolerance of hosts to GINs and other pathogens. This is practised most commonly in the dairy industry, with testing of bulk-tank milk samples used to assess herd health and inform disease control strategies (Nielsen *et al.*, 2000; Stabel *et al.*, 2002; Sekiya *et al.*, 2013; Parker *et al.*, 2017). A limitation of antibody quantification in non-milk-yielding individuals is the necessity for an invasive sampling procedure. A second limitation is that different tissues will have a different balance of antibodies due to their source, such that milk antibody levels are not directly comparable to serum antibody levels, and neither necessarily reflect mucosal immunity. There is therefore a need for a non-invasive method of antibody quantification that is applicable to all individuals, irrespective of age, gender and other variables. Furthermore, FAD has the potential to more directly derive information regarding gastrointestinal health than equivalent diagnostics on milk or serum.

For the purposes of animal health investigation, enzyme-linked immunosorbent assays (ELISAs) are typically conducted on plasma, serum or milk samples. However, a small number of studies have utilized animal fecal samples collected in the laboratory (Wedrychowicz *et al.*, 1985) and field (Peters *et al.*, 2004; Watt *et al.*, 2015). Watt *et al.* (2015) specifically measured antibodies of *Teladorsagia circumcincta*, a GIN. While *T. circumcincta* is predominantly a parasite of sheep, there is mounting evidence for the cross-reactivity of antibodies, produced against antigens of a specific nematode, to other GIN species (Blanchard and Wescott, 1985; Molina *et al.*, 1999; Ruma *et al.*, 2016). Immunological results from fecal material are more likely to be representative of gastrointestinal mucosal membranes than those from other tissues such as plasma, due to the physiologically localized nature of immunity (Lamm, 1988; Wennerås *et al.*, 1999), therefore providing novel and complementary information about animal health.

There is the potential for FAD ELISAs to allow for quantitative assessment of the immunological status of gastrointestinal mucosal membranes as a function of general animal health, gut health or GIN challenge. Fecal material is easily and commonly collected for the purpose of FECs. The benefits of FAD and the novel information it can provide makes it a promising technique for the future veterinary, agricultural and zoological studies into animal health.

The primary objective of the research presented here was to determine the feasibility of a cattle fecal supernatant as a suitable material for quantitative detection of antibodies using ELISA. Further, to assess if fecal antibody levels are representative of those at mucosal membranes: spefically, whether IgA is the most abundant antibody in both cases. Finally, to assess whether fecal antibody levels correlate with serum antibody levels and FEC.

Methods

Sample collection and processing

Sample herds

Fecal samples were taken from cattle at three UK beef farms.

Farm #1 was at Rothamsted Research's North Wyke Farm Platform, in Devon, England. The Farm Platform has three non-organic, pasture-fed beef herds, under typical managed rotation. Each herd is similar but grazes on different pasture systems. An initial sampling on 10/11/2016 collected 45 fecal samples and the second sampling on 07/02/2016 collected 18 fecal samples, six of which were from animals sampled the first time around. Both sampling instances occurred during housing when animals were on a locally produced silage diet.

Farm #2 was a pasture-fed beef farm in Hertfordshire, England. Animals were mob-grazed, i.e. frequently moved to new pasture, with movement approximately every 3 days. Sampling occurred once, on 02/02/2017, during housing, when 30 fecal and 22 blood samples were taken from 30 individuals. The farm was organic (soil association certified) and no anthelmintic treatment had been administered during the monitored season.

Farm #3 was a pasture-fed beef farm in Angus, Scotland. Cattle were mob-grazed and moved between fields up to three times per day. Sampling occurred once, on 07/12/2017, and resulted in the collection of fecal samples from 30 animals. Animals grazed year-round with no housing. The farm was organic (soil association certified) and no anthelmintic treatment had been administered during the monitored season.

Blood serum

Tail venepuncture was conducted, by a trained and licensed veterinarian, from 22 individuals on farm #2, to withdraw blood for regulated disease testing; sub-samples were taken for FAD. Blood samples were only collected from animals for which matched dung samples were available, and blood and fecal samples were taken on the same day. Samples were drawn, by sterile syringe, into labelled 10 mL BD Vacutainers® and rested for >30 min to allow for clotting. Samples were then centrifuged at 2500 rpm/1056 × ***g*** (Sorvall SLA-3000 rotor in a Sorvall RC-5B centrifuge) for 15 min and the supernatant serum withdrawn, using sterile pipette tips, into 1.5 mL microcentrifuge tubes (Thermo Scientific™ 3451). Samples were immediately stored at −20 °C until analysis.

Fecal supernatant

A dung supernatant was obtained by the dilution of fresh cattle dung with a protease inhibitor, centrifuging and withdrawal of supernatant.

Fresh dung was collected from individual animals immediately after deposition, using a clean, single-use polystyrene spoon. Dung was homogenized by stirring before collection, with care taken not to mix in foreign matter such as other dung and hay. Collected samples were transferred to sterile polystyrene screw-top containers. During sampling, the samples were stored in a cool box with ice packs, after which they were stored at −20 °C until being processed.

In order to create the supernatant, dung samples were allowed to defrost at room temperature (approximately 3 h). Defrosted samples were thoroughly mixed using sterile inoculating needles (Camlab, UK). Between 2 and 4 g of dung was then transferred to a sterile beaker and mixed with a protease inhibitor (cOmplete, EDTA-free Protease Inhibitor Cocktail, Roche, Basel, Switzerland) at a recorded ratio of between 1:1 and 1:2 (w/v). The resulting mixture was homogenized using sterile inoculating needles and then transferred to sterile 10 mL centrifuge tubes (Oak Ridge High-Speed PPCO, Nalgene, USA) and rested on ice for >10 min, until centrifuging. Samples were centrifuged at 3–6 °C and 8400 rpm/12 000 × ***g*** (Sorvall SLA-3000 rotor in a Sorvall RC-5B centrifuge, ThermoFisher Scientific, Waltham, Massachusetts, USA) for 5 min. The supernatant was then pipetted, using sterile pipette tips, into 1.5 mL microcentrifuge tubes (Thermo Scientific, USA). Samples were immediately stored at −20 °C until analysis.

Three negative control protease inhibitor blanks for the supernatant diluent were created, comprising of 100% protease inhibitor cocktail. Each blank came from a different batch of inhibitor cocktail and was prepared separately.

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Assay protocol

Seven, bovine-specific ELISAs were conducted. Total IgA, IgG and IgM ELISAs were conducted using bovine-specific commercial components (Bethyl Laboratories Inc., Montgomery, Texas, USA) and a reference serum, per the manufacturer's protocol. A further three ELISAs were conducted using *T. circumcincta* antigen, measuring the responses of bovine-specific IgA, IgG, IgM and IgE to the antigen. No commercial bovine-specific IgE components were available, so a fourth assay was completed using a sheep IgE ELISA. These latter ELISAs were conducted using the same protocol as for the commercial ELISAs with the alteration that the commercial capture antibody was replaced with a *T. circumcincta* antigen, as per (Watt *et al.*, 2015). No IgD antibodies were available for inclusion.

Each ELISA was conducted on all 114 fecal supernatant samples and 22 serum samples. Each of the total Ig plates contained a 10-point dilution series of reference material and two or more blanks of TBST (Tris-buffered saline with Tween20 at 0.05%), representing the sample diluent. Three protease inhibitor blanks were also included in each assay. The *T. circumcincta* assays do not have a reference serum available, so had a known positive sample included twice, which showed that the assay worked on that day. The positive control was serum from sheep that had been trickle infected with *T. circumcincta* and had confirmed antigens against L3 *T. circumcincta*, as per (Watt *et al.*, 2015).

Sample dilution

Supernatant and sera had to be diluted to ensure that optical densities (ODs) were within the detection limits set by the sigmoidal curve. Samples were serial diluted and six concentrations (later narrowed down to three) taken forward for use in assays. For each assay and material, one dilution was chosen across all samples as the one to derive results from (see Supplementary material).

Laboratory procedure

Ninety-six-well plates (Nun-Immuno MicroWell MaxiSorp, ThermoFisher Scientific) were coated with 50 μL of the matched rabbit anti-bovine antibody, diluted to 2 μg mL^{-1} in 0.06 M carbonate buffer. For the *T. circumcincta* assays, the coat was *T. circumcincta* L3 somatic antigen at 2 μg mL^{-1} in 0.06 M carbonate buffer. Plates were then covered in a cling film, and stored for 1–3 days at 4 °C prior to use. Plates were removed from the refrigerator and washed 3× in TBST. Meanwhile, samples were defrosted at room temperature (approximately 1 h) and then serial diluted in 2 mL deep-well plates. Fifty microlitres of the appropriate sample dilutions were pipetted into the relative wells on the plate. TBST and protease inhibitor-negative controls were then added. For total antibody assays, the serial dilutions of reference serum were added, acting as a positive control, but also providing concentration curves for later interpolation. For *T. circumcincta* assays, a known positive sheep sample was used as a plate-positive control on the four assays. Plates were then covered in a cling film and incubated for 1 h at 37.5 °C.

Plates were removed from the incubator and washed 5× in TBST. Fifty microlitres of the appropriate rabbit anti-bovine HRP-conjugated antibody was added to each plate (excluding for the *T. circumcincta* IgE assay). No direct HRP-conjugated antibody was available for the *T. circumcincta* IgE assay and instead 50 μL of mouse anti-ovine IgE (monoclonal IgG1) at 10 μL mL^{-1} with TBST was added. *Teladorsagia circumcincta* IgE plates were then incubated for 1 h at 37.5 °C, washed 5× with TBST and then 50 μL of goat anti-mouse IgG1-HRP detection, at 0.125 μg mL^{-1} with TBST, was added. All plates were then covered in a cling film and incubated for 1 h at 37.5 °C.

After incubation, plates were washed 5× in TBST. One hundred microlitres of TMB substrate (KPL, Gaithersburg, Maryland, USA, SureBlue™ TMB Microwell Peroxidase Substrate – single component) was added to each well, plates were then incubated, in darkness, for 5 min at 37.5 °C. Plates were removed from the incubator and 100 μL of the stop solution, 1.0 M HCl, was added to each well (the addition of HCL inhibits enzyme activity and changes the wells from blue to yellow). Plates were immediately read by a plate reader at 450 nm, providing the OD for each well.

Interpolation and adjustment

For each assay quantifying abundances of a total antibody class, the 10-point dilution series was graphed as a sigmoidal curve of OD and antibody concentration. Sample ODs were interpolated onto this curve to generate an antibody concentration for each sample. These concentrations were then adjusted to account for two instances of *in vitro* sample dilution, which occurred initially when fecal supernatants were formed and again during serial dilutions of supernatants. This generated the final concentration of antibody in each fecal sample.

Due to the lack of reference material available for *T. circumcincta*-specific antibody assays, it was not possible to interpolate the results to generate an exact concentration. Instead, a relative scale was created, using the positive control, to allow for simple comparison of samples relative to one another. The value given to each sample was derived from equation (1). As per total antibody class assays, results were then adjusted to account for *in vitro* dilution. In the event that negative values were obtained (i.e. if sample OD was less than TBST OD), values were converted to zero.

$$= \frac{\text{sample OD} - \text{TBST OD}}{\text{positive control OD} - \text{TBST OD}}. \quad (1)$$

Equation (1) – Formula used to generate a relative and arbitrary scale for *T. circumcincta* antibody levels.

Validation

Reference material was essential to confirm the validity of assays and to calculate antibody levels. Total IgA, IgG and IgM reference material was present on each plate of their matched assay. Reference material stock concentrations for total IgA, IgG and IgM were: 0.11, 24 and 1.8 μg μL^{-1}, respectively. Twenty-six dilutions of reference materials were formed using halving serial dilutions. The initial dilution wash 80 μL of reference material with 920 μL of TBST. Seven hundred microlitres of that solution was then withdrawn and added to 700 μL of TBST and the process repeated to form a series of up to 26 dilutions, of which 10 were chosen for each assay (see Supplementary material). Chosen dilutions were based on the past experience of similar assays, which were then tested to ensure suitability, by visually assessing if they produced sigmoidal curves. Before experimental assays were conducted, plates were run with the specified dilutions of reference materials to confirm that the generated curves were suitable and within the detection limits of the assay and plate reader, each assay was repeated five times and plates included two blanks of TBST. As no *T. circumcincta* antibody reference material was available, and therefore could not be quantified, results were measured in relation to other samples. However, the *T. circumcincta*-positive controls were available and used to confirm that the assay worked.

Fecal egg counts

FECs were conducted on all fecal samples used in the ELISA assays. In addition, each farm had FECs conducted in the grazing

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season leading up to sampling, with FECs conducted on 10 randomly collected samples on each of the four to seven sampling visits per farm.

FECs were completed in duplicate, using mini-Flotac and fill-Flotac devices (University of Naples Federico II, Naples, Italy) (Cringoli *et al.*, 2010; Bosco *et al.*, 2014), in accordance with manufacturer methods (5.0 g of faces in 45 mL of flotation solution), giving an analytic sensitivity of five eggs per gram (epg). A flotation solution of 1.34 g mL^{-1} zinc sulphate in deionized water was used. Total eggs counted across both wells of the mini-flotac plate were multiplied by five to determine epg.

Statistical analysis of antibody results

Validations

Assay validity was confirmed using reference material results. For total IgA, IgG and IgM assays, ODs from the 10-point dilution curves were plotted and assays considered valid if sigmoidal curves were produced by the data plots. For *T. circumcincta* assays (for which no reference material was available), the assay was considered valid if the positive controls were significantly higher than TBST blanks, as determined by a two-sample *t*-test.

For fecal supernatants to be validated as a suitable medium for Ig assays, ODs of fecal supernatants, for at least one antibody type, must be higher than that of blanks. This was determined through two-sample *t*-tests individually comparing fecal supernatant antibody levels to those of TBST and fecal supernatant-negative controls.

Antibody levels

One-way analyses of variance (ANOVAs) with Tukey tests were used to determine if antibody concentrations differed between fecal and serum samples and if concentrations of different antibodies differed within samples. This was implemented for both the total antibody assay dataset and for the *T. circumcincta* dataset independently. Initial tests were conducted on both serum and fecal antibody levels combined, to determine differences in antibody concentrations and levels between the two materials.

Fecal and serum data were then split and the tests conducted to identify differences in the abundance of the different antibody types within those two datasets. Due to a large number of negative samples, zero value results were removed from *T. circumcincta* datasets and ANOVAs repeated.

For both the fecal supernatant and serum datasets, Pearson's correlations were performed on all antibody pairings to determine the correlations between antibody classes and subtypes. Further correlations were then conducted to compare the levels of the same antibodies between blood and fecal samples taken from the same animal on the same day. Bonferroni adjustments were made to account for multiple comparisons. Further correlations were also performed on 21 paired serum and fecal supernatants, derived from the same animal, on the same day. A final set of correlations were performed to determine if any antibody types correlated with FEC results (total nematode epg).

Results

Validation of protocol

Assay validation

Ten-point dilution series for each total IgA, IgG and IgM all produced sigmoidal curves (Fig. 1).

Positive controls for the *T. circumcincta* assays yielded consistent and significantly higher ODs than the negative controls (Fig. 2). These differences were confirmed by two-sample *t*-tests for each *T. circumcincta* antibody, IgA ($T = 25.29$, $P < 0.0005$), IgG ($T = 16.44$, $P < 0.0005$), IgM ($T = 17.79$, $P < 0.0005$) and IgE ($T = 35.39$, $P < 0.0005$).

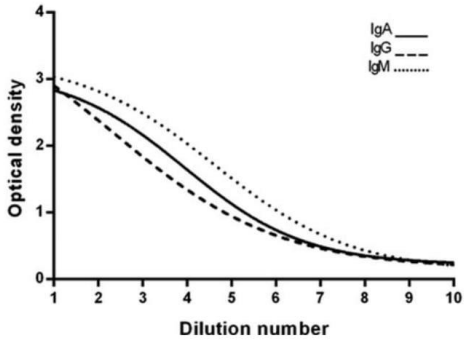

Fig. 1. Sigmoidal curves generated from 10-point dilution series of reference material for IgA, IgG and IgM assays (total and subtype).

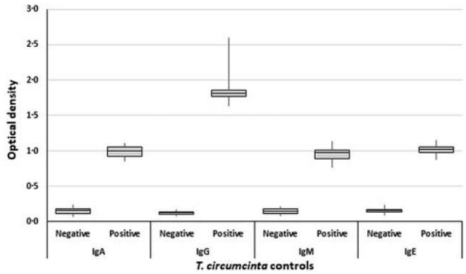

Fig. 2. Boxplots comparing negative and positive controls for all *Teladorsagia circumcincta* assays, for the purpose of validating the ELISA.

Fecal supernatant validation

Fecal supernatant OD values were generally greater than those of TBST and protease inhibitor-negative controls (Fig. 3). The exceptions were *T. circumcincta* IgA, which was not significantly higher than its protease inhibitor control, and *T. circumcincta* IgE, which was not significantly higher than either of its blanks.

Antibody levels

IgA was the most abundant antibody isotype in fecal samples, while IgG was the most abundant antibody isotype in serum samples.

Total antibody concentrations of positive samples varied greatly (Fig. 4). A one-way ANOVA, with a *post hoc* Tukey test, across all total antibody datasets, found that serum antibody concentrations were significantly higher than fecal antibody concentrations ($F = 162.21$, $P < 0.0005$). A second one-way ANOVA and Tukey test, comparing just fecal antibody concentrations, found fecal IgA concentrations to be significantly greater than serum IgG and IgM, which were not significantly different to one another according to the Tukey test ($F = 50.60$, $P < 0.0005$). A final one-way ANOVA and Tukey test, solely comparing serum antibody concentrations, found that serum IgG concentrations were significantly greater than serum IgA and that both were significantly greater than serum IgM ($F = 18.97$, $P < 0.0005$).

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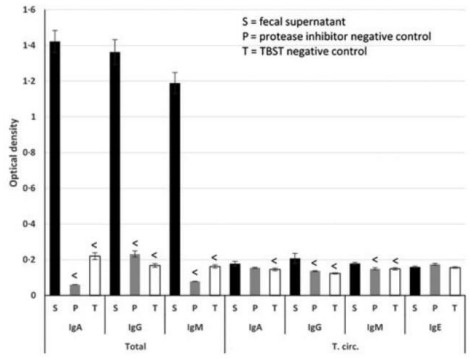

Fig. 3. Unadjusted, 'raw' optical densities for fecal supernatant, protease inhibitor-negative controls and TBST-negative controls, across all assays. Less than symbols (<) above control columns signify that their ODs are statistically significantly less than the sample ODs for the same antibody, as determined by a two-sample *t*-test.

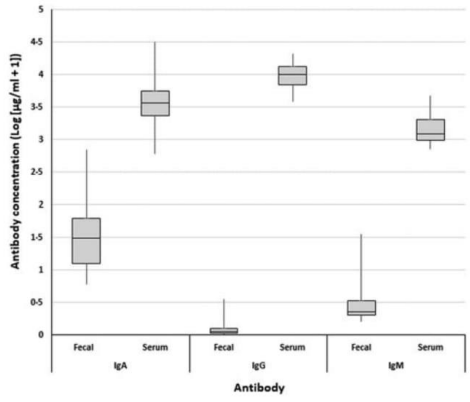

Fig. 4. Boxplots of total antibody concentrations [log (μg/mL +)] measured across all cattle fecal and serum samples.

The variation in *T. circumcincta* antibody levels was much less pronounced than for total antibody concentrations. A one-way ANOVA with a *post hoc* Tukey test found that *T. circumcincta* IgG levels were significantly greater than all other *T. circumcincta* antibodies in the combined dataset of feces and serum, all of which were not significantly different to one another ($F = 548.06$, $P < 0.0005$). These trends were still apparent when the fecal and serum datasets were isolated and analysed independently ($F = 2.11$, $P = 0.098$ and $F = 152.46$, $P < 0.0005$, respectively). However, when only positive samples were included in the analysis, an ANOVA and Tukey test on fecal samples found *T. circumcincta* IgA to be significantly greater than the other antibodies, which were statistically not different to one another ($F = 4.00$, $P = 0.008$) (Fig. 5).

Fecal nematode egg counts

On farm #1, 29% of animals had GIN eggs in their feces; among these animals, the mean epg was 17 (S.E. 7.7). On farm #2, 17% of animals were positive, with a mean epg of 139 (S.E. 82.6). Farm #3 had 27% of animals recorded as positive, of which the mean epg was nine (S.E. 1.8).

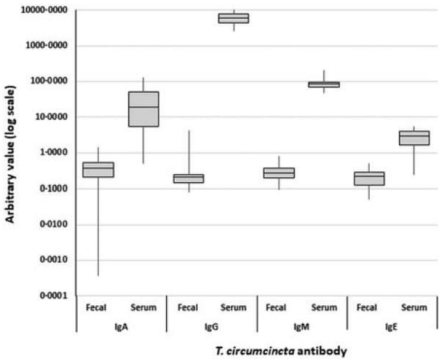

Fig. 5. Boxplots of *Teladorsagia circumcincta*-specific antibody levels (arbitrary units) measured across all fecal and serum samples. *Y*-axis is a log scale.

Correlations

Antibody correlations

The majority (14/21) fecal antibody correlations were found to be significant (Table 1). Of the seven non-significant correlations, five were for pairings that included *T. circumcincta* IgE.

Of the 21 correlations conducted, only one serum antibody pairing (IgM *vs T. circumcincta* IgM) correlated significantly (Table 2).

When comparing levels of the same antibody taken from fecal and serum samples of the same individuals on the same day, the only significant correlation found was with IgA concentrations (Table 3).

Antibody vs FEC

FECs correlated negatively with all antibody types; however, correlations were all <0.1 and non-significant.

Discussion

Assay validity

The experiment achieved its primary objective, to quantify antibody levels in cattle feces, and is therefore considered a valid protocol. Results from reference materials and controls provided sufficient evidence that the commercial ELISA products worked effectively, providing a stable foundation from which to assess the validity of the protocol. Positive controls for the *T. circumcincta* assays also provided evidence that they too worked effectively.

The greater mean ODs observed from fecal supernatants, compared with the blanks of TBST or protease inhibitor, support the validity of a fecal supernatant as a suitable material for ELISAs. This highlights the potential for fecal material to be used in the immunological assessment of animal health, particularly cattle and other ruminants.

Interpretation

Across fecal samples, levels of IgA, both total and *T. circumcincta* specific, were significantly higher than those of all other antibodies. This result is consistent with the literature, that IgA is by far the most abundant antibody at mucosal membranes (Hughes *et al.*, 1981; Lamm, 1988; Macpherson *et al.*, 2008). This finding supports that fecal antibody levels are indicative of mucosal membrane antibody levels, as seen in humans (Crabbé

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Table 1. Pearson's correlation results for different antibodies measured within fecal samples

	IgA	IgG	IgM	*T.* IgA	*T.* IgG	*T.* IgM	*T.* IgE	
IgA		0.562*	0.700*	0.300*	0.651*	0.514*	−0.050	Correlations
IgG	<0.0005*		0.453*	0.161	0.345*	0.238	−0.203	
IgM	<0.0005*	>0.0005*		0.485*	0.640*	0.530*	−0.180	
T. IgA	0.001*	0.085	<0.0005*		0.781*	0.622*	0.079	
T. IgG	<0.0005*	<0.0005*	<0.0005*	<0.0005*		0.654*	0.011	
T. IgM	<0.0005*	0.007	<0.0005*	<0.0005*	<0.0005*		0.364*	
T. IgE	0.593	0.028	0.053	0.402	0.905	<0.0005*		
				P values				

The top right half of the chart shows the correlation statistics and the bottom left half shows the *P* value. Results with an asterisk (*) are statistically significant at an adjusted critical *P* value of 0.0024. '*T.*' refers to *Teladorsagia circumcincta*. $n = 116$.

Table 2. Pearson's correlation results for different antibodies measured within blood samples

	IgA	IgG	IgM	*T.* IgA	*T.* IgG	*T.* IgM	*T.* IgE	
IgA		0.487	0.418	0.161	−0.138	0.103	0.359	Correlations
IgG	0.021		0.500	0.530	0.352	0.324	0.141	
IgM	0.053	0.018		0.439	0.268	0.669*	0.445	
T. IgA	0.473	0.011	0.041		0.235	0.370	0.155	
T. IgG	0.541	0.108	0.227	0.291		0.545	−0.110	
T. IgM	0.650	0.141	0.001*	0.090	0.009		0.014	
T. IgE	0.101	0.131	0.038	0.490	0.625	0.952		
				P values				

The top right half of the chart shows the correlation statistics and the bottom left half shows the *P* value. Results with an asterisk (*) are statistically significant at an adjusted critical *P* value of 0.0024. '*T.*' refers to *Teladorsagia circumcincta*. $n = 22$.

and Heremans, 1968; Tomasi, 1970; Baklien and Brandtzaeg, 1975; Bjerke *et al.*, 1986). To confirm this, post-mortem intestinal washes could be utilized to recover mucosal antibodies and other biomarkers (Negrão-Corrêa *et al.*, 1996) for comparison with those found in feces from the same individual. During gut transit, organic material, which later ends up in feces, might accumulate biomarkers from mucosal membranes, making feces a rich resource for the assessment of gut health. The most abundant antibody in serum was IgG, which is also expected given the literature (Fahey and McKelvey, 1965; Hughes *et al.*, 1981). This provides additional reassurance that the various assays accurately and proportionally represent antibody levels in the relevant tissue/material. Similar relative antibody abundances in feces were also observed in sheep by Watt *et al.* (2015).

Only 15% of fecal samples were returned as positive after FEC, providing an inadequate amount of positive data to determine with any certainty, if a correlation exists between nematode egg counts and fecal antibody levels. The negative correlations observed (although non-significant) are consistent with the observations by Watt *et al.* (2015). The lower fecal antibody levels and lack of correlation with FECs may stem from hypobiosis as samples were taken during late autumn and early winter (Capitini *et al.*, 1990). Moreover, observed FECs were rather low. A longitudinal study, tracking seasonal fecal antibody trajectories would clarify this and potentially provide a more suitable FEC dataset for analysis.

Assays for *T. circumcincta*-specific antibodies were only able to provide relative and arbitrary results due to there being no available reference material. The total antibodies standard curves could not be used for interpolation of *T. circumcincta* antibodies as the relative avidities of both capture antibodies is unknown. To achieve quantitative concentrations, a reference sample with a known concentration of the relative *T. circumcincta* antibodies would need to be created. This would require the artificial infection of a host animal (likely sheep), with *T. circumcincta*, followed by slaughter and measurement of antibody concentrations in the blood, which was not a viable option in the current work.

Application

The absence of a correlation between blood and fecal antibody levels shows that the method is not a replacement or proxy for measurements of systemic antibody levels. However, results support the utility of FAD to derive specific information about animal health that cannot easily be obtained otherwise. This information may prove to be of greater use and relevance for the assessment of GIN derived, and other, gut damage, than circulating serum antibodies. Similar recent advances have seen the development and adoption of salivary antibody tests, for the study of GIN in sheep (Shaw *et al.*, 2012). The Carla Saliva Test detects Carla antibodies (Harrison *et al.*, 2003) in sheep saliva; however, these antibodies are also present in gastrointestinal mucus, meaning that FAD may be a suitable approach for measuring Carla antibodies. The primary disadvantage of a salivary test, compared with FAD, is the necessity to perform an invasive procedure on a restrained animal. Research and development of FAD methodologies and associated technologies, using advancements on salivary antibody tests as a template, has the potential to create a highly practical and informative diagnostic method.

It is evident that FAD has the ability to quantify symptomatic and important aspects of animal immunology; however, there is

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Table 3. Pearson's correlations comparing levels of the same antibodies from both fecal and serum samples taken from the same individual on the same day

	IgA	IgG	IgM	*T.* IgA	*T.* IgG	*T.* IgM	*T.* IgE
Correl.	0.581*	0.511	0.010	−0.028	−0.207	0.173	0.103
P value	0.005*	0.105	0.966	0.900	0.354	0.442	0.648

Results with an asterisks (*) are statistically significant at an adjusted *P* value of 0.0071. '*T.*' refers to *Teladorsagia circumcincta*. $n = 22$.

limited understanding about what precisely fecal antibody levels indicate, especially in relation to pathogen-driven pathology. Given the multi-functional role of antibodies, FADs may be best used as a general marker of animal gut health and disease challenge, particularly from gastrointestinal pathogens such as GINs, and applied as part of a TST strategy. Larger scale and more longitudinal studies are necessary to further understand how FAD could best be utilized.

Detection of molecules within feces need not be restricted to antibodies, and there are a range of biomarkers to which the outlined protocols might be adapted. Two prime candidate molecules are the inflammatory markers: lactoferrin and calprotectin, which are routinely quantified within human medicine, for the diagnosis of bowel diseases (Røseth *et al.*, 1999; Lundberg *et al.*, 2005; Gisbert *et al.*, 2009; Lamb and Mansfield, 2011). Furthermore, gut inflammation can be symptomatic of GIN damage. Lactoferrin is monitored in milk as part of quality and safety assurance, therefore bovine assays are commercially available. Pepsinogen and gastrin ELISAs can be used as veterinary immunodiagnostic tools for GIN infections and are therefore also strong candidates for fecal detection, given their established utility as immuno-markers (Berghen *et al.*, 1993; Charlier *et al.*, 2011).

The outlined protocols produce a large amount of fecal supernatant, providing enough for multiple assays. Once protocols have been developed, throughput can be extremely high; within this study, for example, sixteen 96-well plates could be completed manually within one day. This number could be increased, for example, with automated pipetting machines. This brings about the possibility of fecal supernatants being used to provide a wealth of immunological data, paired with other measures of animal health, as part of a comprehensive and longitudinal animal health assessment, driving highly targeted individual interventions to support efficient and sustainable disease control.

In conclusion, the results presented advance the potential of animal feces as a resource for veterinary diagnostics. Consistent positive Ig levels, above background levels, combined with the range and distribution of results, support the methodology as a valid immunological tool. Results indicated that fecal antibody levels are representative of gastrointestinal immunology, due to the similarity in antibody profiles of fecal material compared with those observed at mucosal membrane surfaces, with IgA being the most abundant antibody (Lamm, 1988; Mazanec *et al.*, 1993; Macpherson *et al.*, 2008). This is also in-keeping with the passage and processing of material through the gut and into feces. Therefore, FAD has the potential to provide novel and unique information about gastrointestinal health and immunology.

FAD is a new, but promising, capability to assess immunological aspects of ruminant gut health in a timely and cost-effective manner. The method is highly ethical as it is non-invasive, which brings the additional benefit of not requiring trained veterinarians or licensing under animal protection legislation. For more comprehensive interpretation of fecal antibody levels, further work needs to be performed to determine the drivers of fecal antibody concentrations, most notably the role of pathogens. Successful FAD protocols within this study and by Watt *et al.* (2015) suggest that FAD might be more widely applicable to other mammals, particularly ruminants. Further advancements in the detection of fecal immuno-markers could, in the future, become part of a comprehensive tool kit for the assessment of animal health and development of disease prevention strategies.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0031182018000902.

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Conflict of interest. None.

Ethical standards. Blood sampling was covered by the Veterinary Surgeons Act 1966. Blood samples were taken by a qualified veterinarian with the approval of the owner. Samples were taken for regulated and scheduled animal health testing. Sub-samples were taken for the purpose of this study as and when blood, in excess to that required by the veterinarian, was available. All results were fed back to the veterinarian as per their request.

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